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INFLUENCE OF FERROUS IRON ON THE AEROBIASIS OF *CORYNEBACTERIUM DIPHTHERIAE*

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IN a study concerning a metabolite of *Corynebacterium diphtheriae*, it was necessary to procure from reliable sources strains of toxigenic *C. diphtheriae*. Received on Loeffler's medium or agar slants, these strains failed to show the characteristics of the aerobic prototypes, such as pellicle formation in peptone infusion broth, pH balance and toxin production in amino-acid media (Mueller and Miller, 1941). Since Pappenheimer and Johnson (1936) and Pappenheimer and Hendee (1947) demonstrated that synthesis of iron resulted in greater concentration of aerobic respiratory pigment (cytochrome *b*), a study was undertaken to determine the influence of ferrous iron on the reconversion of the above strains to the aerobic state and to toxigenicity.

MATERIALS AND METHODS

Cultures studied.—Numbered cultures 9343, 9673, 9674, 9675, and strain Park 8 were obtained from the American Type Culture Collection, Washington, D.C. Prototypes C. Mitis, Gravis and Intermedius were obtained from the U.S. Public Health Service Laboratories, Chamblee, Ga.

Media.—Mueller and Miller casamino acids medium was made in accordance with original instructions using " Difco " Casamino acids as the hydrolysate (Mueller and Miller, 1941). To stimulate aerobic oxidation 0·7 ml. of sodium lactate and 0·6 g. sodium succinate $6H_2O$ were added to 200 ml. of medium (Yoneda, 1957). Veal infusion peptone broth was made from an infusion of " Bacto-Veal " (Difco) to which was added 1 per cent proteose-peptone (Difco) and 0·4 per cent sodium chloride. Phenol red was added to all liquid media in final concentration of 0·6 mg. per cent, except in those instances where iron assays were performed. The starting pH of all cultures was within the range 7·6–7·8.

Chemical procedures.—Deferration of Mueller and Miller casamino acids, solution No. 1, solution No. 2 and maltose was in accordance with the work of Mueller and Miller (1941). Deferration of veal infusion peptone broth was accomplished in the same manner, except for the substitution of lesser quantities of disodium phosphate and calcium chloride (Drew and Mueller, 1951). Ferrous and total iron were determined colorimetrically, based on the complex formed between O-phenanthroline and ferrous iron. For this purpose the general procedure of Saywell and Cunningham (1937) was used, with elimination of the digestion step, since our assays were concerned with free iron inherent in or that added to media,

rather than that which was organic bound. A standard curve of from 1–10 μg. was derived from the coloured complex formed by O-phenanthroline with reduced electrolytic iron. Readings were made at 510 mμ in a Coleman Spectrophotometer.

Toxin estimation.—Toxin contents of media were estimated by the Ramon flocculation test. Reagents used were, Lederle Purogenated Diphtheria Toxoid 1160 LF/ml., Lederle Diphtheria Flocculating Serum 16D6, 500LF/ml. and NYC Dept. of Health Diphtheria Antitoxin 1173, $\pm$700 LF/ml.

RESULTS

All cultures initially received on agar slants or Loeffler's coagulated medium were maintained on Loeffler's medium (Difco). They were transferred to non-assayed veal infusion peptone broth with and without maltose to determine their abilitites to produce pellicle formation after 48–72 hr. incubation at 34–35°.

Table I presents data to demonstrate that pellicles were not produced, and that pH balance could not be obtained within the concentration of maltose (1·4 per cent) used by Mueller and Miller (1941) for toxin production.

TABLE I

Culture	Maltose 1·4 per cent	Maltose 1 per cent	Maltose 0·5 per cent	Maltose 0 per cent
9675	AC	SL. AC	ALK N.P.	ALK N.P.
9673	AC	AC	Neut. N.P.	ALK N.P.
9674	AC	AC	AC	ALK N.P.
Park 8	AC	AC	AC	ALK N.P.

AC = acid, SL = Slight, Neut. = Neutral, ALK = Alkaline, N.P. = No pellicle, P = Pellicle.

Mueller and Miller (1941) ascribed a reciprocal relationship to the concentrations of amino acids with that of maltose necessary to provide proper pH balance and toxin production. Consequently the above experiment was repeated with substitution of Mueller and Miller's medium for veal infusion peptone broth. The medium was deferrated and adjusted to contain the optimal amount of nitrogen of 0·26 per cent and 5 μg. Fe++ was added per 20 ml. This concentration of iron was shown by Pappenheimer and Johnson (1936), to be within the range of minimal toxin production.

Table II demonstrates that with fixed quantities of amino acid nitrogen and iron, and with varying quantities of maltose, pellicle formation and pH balance could not be obtained with 3 of the 4 strains tested.

TABLE II

Culture	Maltose 1·4 per cent	Maltose 1 per cent	Maltose 0·5 per cent	Maltose 0 per cent
9675	AC	AC	AC	ALK N.P.
9673	AC	AC	AC	ALK N.P.
9674	AC	AC	AC	ALK N.P.
9343	AC	Neut. P.	Neut. P.	ALK N.P.

If the reaction of culture 9343 was a result of nitrogen-maltose balance then it was possible for the other 3 cultures to react similarly by finding the proper nitrogen-maltose ratio. Therefore the above experiment was repeated using varying quantities of amino-acid nitrogen combined with varying amounts of maltose, ferrous iron (5 μg./20 ml.) remaining constant in all flasks.

The results in Table III indicate that the organisms *per se* were deficient in a

substance necessary to regulate the rate of metabolism of both maltose and amino acids.

TABLE III

Cult.	0·26 per cent N Maltose (per cent) 1·25	1·0	0·75	0·33 per cent N Maltose (per cent) 1·25	1·0	0·75	0·43 per cent N Maltose (per cent) 1·25	1·0	0·75
9673	AC	AC	AC	AC	AC	AC	AC	AC	AC
9674	AC	AC	AC	AC	AC	AC	AC	AC	AC
9675	AC	AC	AC	AC	AC	AC	AC	AC	AC

That ferrous iron in adequate concentration is the substance required by the organisms to regulate their metabolism of both maltose and amino acids, is demonstrated in Table IV. To deferrated veal infusion broth, varying amounts of ferrous iron were added to give the concentration listed. Maltose, 1·4 per cent remained constant in all flasks containing 20 ml. of medium. Inoculations were made from cultures maintained on Loeffler's medium.

TABLE IV.—*Concentration of Fe++ μg. per 20 ml.*

Cult.	2	4	6	8	10	12	14
Park 8	AC	AC	AC	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.
9674	AC	AC	AC	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.
9343	AC	AC	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.
Gravis	AC	AC	AC	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.
Mitis	AC	AC	AC	SL.ALK.N.P.	SL.ALK.N.P.	SL.ALK.P.	SL.ALK.P.
Interm.	AC	AC	AC	SL.ALK.N.P.	SL.ALK.P.	SL.ALK.P.	SL.ALK.P.

It is apparent that at some given concentration of iron, pellicle formation and pH balance occur and persist beyond that concentration, whereas concentrations less than the minimum requirement show lack of pellicle formation and show acidity.

Attention is drawn to the fact that culture 9343, the only strain in Table II to produce a pellicle, did so because the medium in that experiment contained 5 μg. Fe++/20 ml. which approximates its minimum requirement of 6 μg.Fe++/20 ml. as shown in Table IV.

It was of interest to note to what extent the various strains of *C. diphtheriae* utilized the iron available to them in the medium. For this purpose, veal infusion peptone broth of high iron content was inoculated with several strains of *C. diphtheriae* that had been maintained on Loeffler's medium. All flasks prior to inoculation contained by assay 19·2 μg. of ferrous iron/20 ml. and 1·4 per cent maltose. The starting pH was 7·65. Since there was a 10 per cent loss of fluid volume during the 7 day incubation period at 34–35°, assay results on the centrifuged broths were computed on the basis of 18 ml. rather than 20 ml.

The results from Table V show that Gravis, Intermedius and P8 limit their depletion of iron to between 30–40 per cent of the total in a 7 day period, whereas Mitis depletes the iron content by 71 per cent in the same period of time. When comparison is made of the actual amount of iron removed from the medium in this experiment and the minimal requirement for each strain for pellicle production and pH balance (Table IV), it can be seen that they closely approximate each other. Therefore in this system the cultures limit utilization of iron, and all amounts above the minimal for pellicle formation and pH balance are in excess.

TABLE V

Culture	Final pH	Fe++ depleted μg./20 ml.	Fe++ remaining μg./20 ml. assay	Per cent depleted
Control broth	7·65	—	19·2	0
Gravis	7·80	5·7	13·5	30
Mitis	7·60	14·7	4·5	71
Intermedius	7·30	7·7	11·5	40
Park 8.	7·80	5·7	13·5	30

It was of further interest to determine whether transfer from high maintenance iron medium to high starter iron medium would result in the same degree of iron utilization that occurred when the transfer was from Loeffler's to high iron medium. For this study, cultures were maintained through several passages on high iron veal infusion peptone agar slants (1 μg./ml.) and then inoculated into fluid media of equal iron content. The same degree of iron utilization no longer occurred, as shown in Table VI.

TABLE VI

Culture	Final pH	Fe++ depleted μg./20 ml.	Fe++ remaining μg./20 ml.	Per cent depleted
Control broth	7·80	—	23·6	0
Gravis	8·20	15·7	7·9	66
Mitis	8·00	17·1	6·5	72
Intermedius	7·85	15·5	8·1	66
P8	6·70	17·1	6·5	72

It seems reasonable to assume that the concentration of iron in maintenance media influences the ability of the growing organism quantitatively to utilize iron upon subsequent transfer to other media. In this respect, the Loeffler's medium by assay had a ferrous iron content of 0·3 μg./ml. in the liquid state, and probably less than this amount was available to the organism on the coagulated slant; whereas the agar slants contained more than 3 times the quantity of ferrous iron.

Having determined that aerobiosis could be induced in high iron "starter media" the question now arose as to whether the pellicle from this medium was capable of producing toxin in media containing various iron concentrations. For this purpose, the 2 cultures selected were prototypes Mitis and Gravis, each having shown different minimal requirement for iron in "starter medium". These cultures maintained on Loeffler's medium were inoculated into veal infusion peptone broth containing 1·4 per cent maltose, approximately 20 μg. ferrous iron per 20 ml. with a pH of 7·6 and after 72 hr. incubation, large pieces of pellicle were transferred to Mueller and Miller's amino acid medium containing various quantities of iron. After 7 days incubation, the media were clarified by high speed centrifugation and filtration, and tested for toxin. Table VII shows that both types produced toxin, Mitis in higher concentration and through a wider range of iron than did Gravis.

DISCUSSION

Customarily, the first empirical step taken in toxin production is the development of a pellicle in a non-assayed (iron) starter medium. Our results indicate that the failure or success of this initial procedure is directly related to the absence or

TABLE VII

Mitis		Gravis	
Fe++ μg./20 ml.	Toxin LF/ml.	Fe++ μg./20 ml.	Toxin LF/ml.
3·2	72	3·2	50
4·2	56	4·2	15
6·2	45	5·2	10
8·2	35	6·2	0
10·2	0	7·2	0
14·2	0	8·2	0

presence of sufficient iron in the starter medium. Where there is a deficiency of iron in the latter, cultures will fail to show aerobic characteristics, but if these cultures are transferred to a medium containing iron in sufficient quantity they will build up their aerobic respiratory systems. That the iron depleted from the medium is assimilated by the bacterial cell has already been established (Pappenheimer, 1955). Concomitant with adequate utilization of such iron, pellicle formation and pH balance occur, and these characteristics persist upon transfer of the pellicle to iron deficient media for toxin production.

Our results further indicate that the amount of iron required in medium for toxin production can be predetermined for any particular strain, and that this amount must be less than that which was initially required by the organism for establishment of aerobic growth. Thus, Mitis when transferred from low iron stock medium (Loeffler's) to high iron broth, becomes aerobic at 12 μg. iron per 20 ml. of medium, and when the pellicle thus formed is transferred to other media containing less than this quantity of iron, toxin is produced. Similarly, Gravis becomes aerobic at 8 μg. of iron per 20 ml. of medium and when the pellicle is transferred to other media containing less than 8 μg., toxin is produced. The principle involved here in toxin production follows Pappenheimer's concept of the breakdown of respiratory pigment in a medium where there is insufficient iron to synthesize the protein (toxin) and porphyrin moieties to cytochrome, thus resulting in their liberation into the medium. Our contribution to this concept is that the breakdown of respiratory pigment is not possible unless sufficient pigment is first synthesized in adequate concentration for its eventual breakdown.

SUMMARY

The underlying cause for the failure of *C. diphtheriae* to grow aerobically under certain conditions has been determined, and a method has been presented by which such failure can be prevented. In order to develop its cytochrome system, for aerobic growth, the diphtheria bacillus must have available to it a certain minimum of ferrous iron, which is measurable, and which if added to the culture medium will ensure aerobic growth.

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STUDIES OF *TOXOPLASMA GONDII* IN CELL CULTURES BY MEANS OF IRRADIATION EXPERIMENTS*

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OBSERVATIONS on the multiplication of *Toxoplasma gondii* in different cell types of human origin were described in previous reports (Sourander, Lycke and Lund, 1960 ; Lund, Lycke and Sourander, 1961). The present report deals with observations of parasite-cell interactions after irradiation of cell cultures and parasites prior to the inoculation and the effect of the irradiation on parasite-infected cells. This was done in order to see whether inhibition of mitotic activity affected parasite propagation.

MATERIAL AND METHODS

The parasites used were obtained from freshly harvested peritoneal exudate of Swiss albino mice infected with the RH strain of *Toxoplasma gondii*.

The cell cultures were HeLa or Detroit-6 cells cultures in Gey chambers as described previously. The medium was Hanks' balanced salt solution to which was added 10 per cent human or calf serum, which was dye test negative, 0·5 per cent lactalbumin hydrolysate, 100 I.U. penicillin, and 100 μg. streptomycin. The cells were carefully rinsed 2 hr. after the inoculation of the parasites and the medium was renewed to avoid toxic effects of the mouse exudates on the cell cultures. The cultures were incubated at 37° and the medium was replaced with fresh medium every second or third day. Infected cultures were examined daily in a phase contrast microscope and photographs were taken.

The dye tests were performed according to the methods described by Sabin and Feldman (1948). As source for accessory factor fresh human sera were accepted if they were dye test negative and enabled a positive reference serum to react with a constant dye test titre in repeated tests.

The antiserum used was either a human serum, which was positive in the dye test in dilutions of 1/8000 or more or a rabbit hyper-immune serum also giving a dye test titre of 1/8000. The rabbit sera were produced by intravenous inoculation of rabbits with 0·1 ml. of a mouse exudate diluted 1/100. Irradiated and non-irradiated toxoplasma parasites were tested simultaneously as antigens to obtain titres against the reference antisera.

Irradiation technique.—In preliminary experiments the irradiation was performed by means of a conventional 250 kV X-ray unit, parasites and cells were exposed to doses varying from 600–3000 r. Later gamma rays from a 1·5 MV. ^{60}Co source were used. With this method doses varying from 30,000–120,000 rad were employed.

RESULTS

In a first series of experiments the toxoplasma parasites were irradiated. The parasite-containing peritoneal mouse exudate was divided in two portions. One portion was irradiated and the other served as a non-irradiated control. Both were used as dye test antigens and also inoculated into groups of cell cultures.

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When 600–3000 r of X-ray irradiation were given irradiated parasites did not behave differently from non-irradiated ones with regard to infection and multiplication rates as well as antigenic properties in the dye test. The X-ray unit could not be used for exposures longer than 30 min. and therefore doses exceeding 3000 r could not be obtained.

On the other hand the high intensity of the gamma irradiation did not allow doses below 30,000 rad to be used if the exposure time should not be inconveniently short. Using the gamma irradiation it was found that the irradiation did not change the stainability of the parasites or the antigenic properties as measured by the dye test. Results of experiments reported previously (Lund, Lycke and Hahn, 1960) indicated that loss of infectivity of parasites due to thermal inactivation is reflected in reduction of the number of stainable parasites. Therefore inactivation of parasites due to heat during the experimental procedure was considered negligible.

Parasites irradiated with 30,000 rad or more penetrated the cells just as untreated ones. But whereas non-irradiated parasites multiplied intracellularly and caused cell burst within 48 hr. the irradiated parasites did not divide. Only in a few cases abortive multiplications of irradiated parasites were found. Clones consisting of more than 4 parasites were never observed. After 14 days cultures inoculated with non-irradiated exudate showed parasites still in active stages of multiplication as revealed by figures of parasite divisions, cells with clones of numerous parasites, bursting cells and free extracellular parasites in the culture fluid. At this time no parasites could be detected in cultures inoculated with portions of the irradiated exudate (Fig. 1 and 2).

Two days old HeLa or Detroit-6 cells were exposed to 600–3000 r. After 48 hr. a number of giant cells were observed in the irradiated cultures. The number of such atypical cells increased gradually. However a great number of cells seemed to be morphologically unchanged. The cultures were infected with toxoplasma 48 hr. after X-ray treatment. Microscopic examinations on the following days revealed no differences between irradiated and non-irradiated cultures with respect to parasite multiplication. The development of parasites in the giant cells proceeded in the same way as in the non-irradiated cells.

When cell cultures were exposed to 30,000–120,000 rad of gamma-irradiation not only inhibition of mitotic activity was observed but grave cell damage was demonstrable already on the day following the irradiation. With 120,000 rad most cells degenerated without giant cell formation. After a couple of days no living cells could be observed in the cultures. Exposure to 60,000 rad caused death of most cells but also some giant cell formation. Such cells survived for a couple of weeks. After exposure to 30,000 rad finally most cells survived. The giant cell formation was very pronounced and these cells could be maintained for at least 4 weeks.

When cells irradiated with the gamma beam were infected with parasites, the multiplication of parasites proceeded at the same rate as in untreated cultures resulting in clone formation and cell rupture. Even the dying cells which had been treated with 120,000 rad allowed parasite multiplication. If parasites formed in irradiated cultures were passed over to new cultures they proved infective. (Fig. 3–5.)

The cultures were irradiated with 30,000 rad 3 hr. or 3 days after the inoculation of parasites. This treatment inhibited the parasite multiplication as well as

the mitotic activity of the cells. The giant cells formed survived for more than 3 weeks but no parasite multiplication was observed in the cells after the irradiation. The parasites that had penetrated or been formed in the cells before the irradiation lost their normal appearance. Thus, no internal organization could be detected, they became rounded with less distinct boundaries, and gradually the single parasites within a clone could not be distinguished. Finally there remained only dark bodies and granules from the disintegrated parasites. (Fig. 6–8.)

DISCUSSION

The electronmicroscopic studies performed by Gustafson, Agar and Cramer (1954) among others, have given evidence for a complex internal organization of the toxoplasma parasite. The parasite has a nucleus containing DNA (Sabin 1942) and a nucleolus. The cytoplasm contains regularly demonstrable structures such as the toxonemes (Gustafson *et al.*, 1954).

The parasite multiplies by binary fission as already described by Cross (1947). Thus it possesses many characteristics of a complete cell.

The mitotic activity of the mammalian cell may be affected by ionizing radiation. The chemical structure of cellular proteins and therefore also the antigenic properties are on the other hand not affected by doses sufficient to inhibit mitotic activity. For such reasons it seemed of interest to study the effects of irradiation on toxoplasma parasites. The experimental results reported showed that high doses of irradiation did not inhibit penetration into the cells but prevented multiplication of the parasites. Inhibition of multiplication was also seen of such parasites as were intracellular at the time of irradiation. However the antigenic properties utilized in the dye test remained unaffected. Thus toxoplasma reacted to irradiation with regard to antigenic properties and mitotic capability in the same way as mammalian cells.

Nothing is known about the reasons for the toxoplasma being an obligate intracellular parasite. It seems however that the intracellular metabolites necessary for parasite multiplication must be amply supplied, because parasite multiplication and growth may proceed without decrease in rate until the cytoplasm of the host cell is completely occupied by parasites (Lund, Lycke and Sourander, 1961).

EXPLANATION OF PLATES.

FIGS. 1–8.—Phase contrast micrographs of living cells.

FIG. 1.—HeLa cells with clones of toxoplasma parasites one week after inoculation of the culture with non-irradiated parasites, ×466.

FIG. 2.—HeLa cells one week after infection with parasites which had been irradiated with 30,000 rad high energy gamma rays prior to inoculation of the culture. No clones are seen, but single parasites are found in cytoplasmic vacuoles, ×500.

FIG. 3.—Detroit-6 cells, ×312.

FIG. 4.—Detroit-6 cells 2 weeks after irradiation with 30,000 rad. Note the pronounced giant cell formation with swelling of the nuclei, ×312.

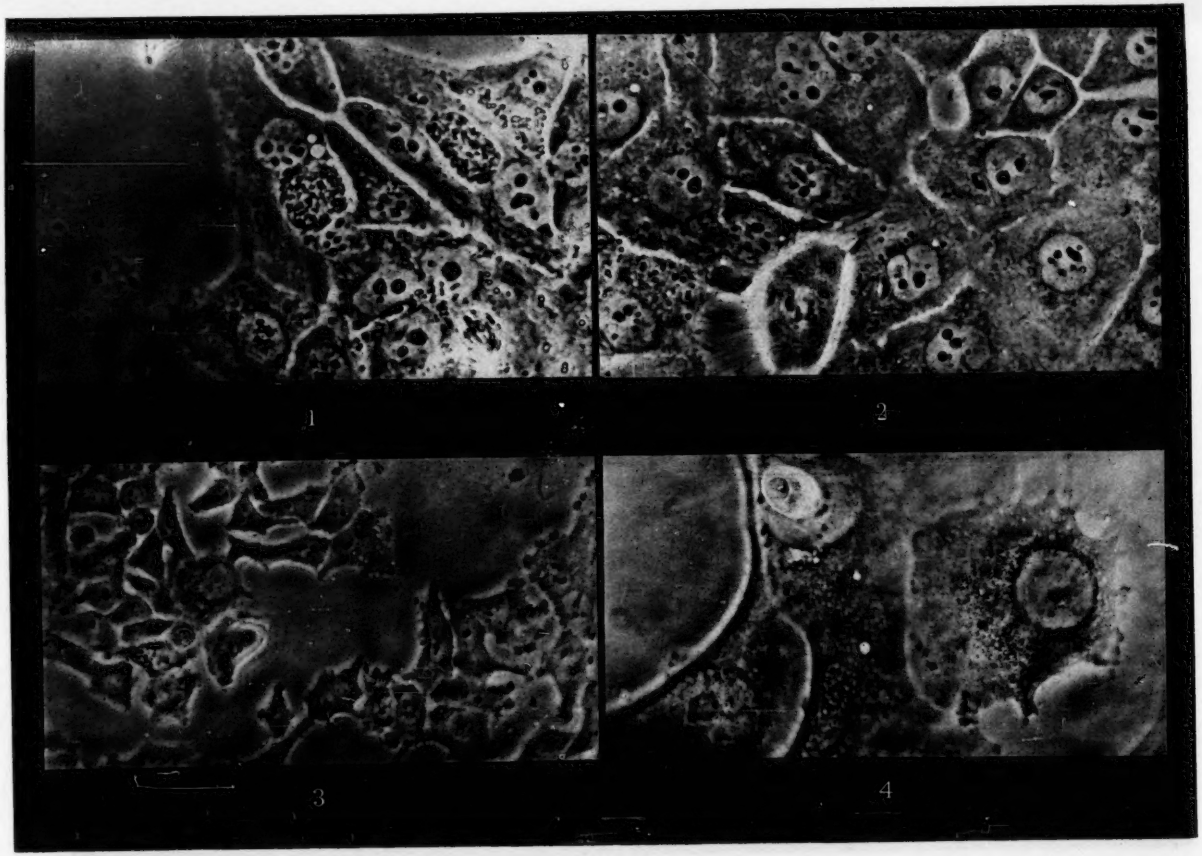
FIG. 5.—HeLa cells 2 weeks after irradiation with 30,000 rad. Many giant cells, some of them with multiple nuclei are seen, ×234.

FIG. 6.—HeLa cells one week after inoculation of the culture with toxoplasma. Many stages of infection are seen including rupture of parasitized cells and reinfection of other cells, ×545.

FIG. 7.—HeLa cells irradiated 3 hr. after infection. Dose 30,000 rad. One week after irradiation atypical parasites are found, but no clone formation, ×545.

FIG. 8.—HeLa cells irradiated 3 days after infection. Dose 30,000 rad. One week after irradiation disintegrated clones are seen, ×545.

BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY. Vol. XLII, No. 5.



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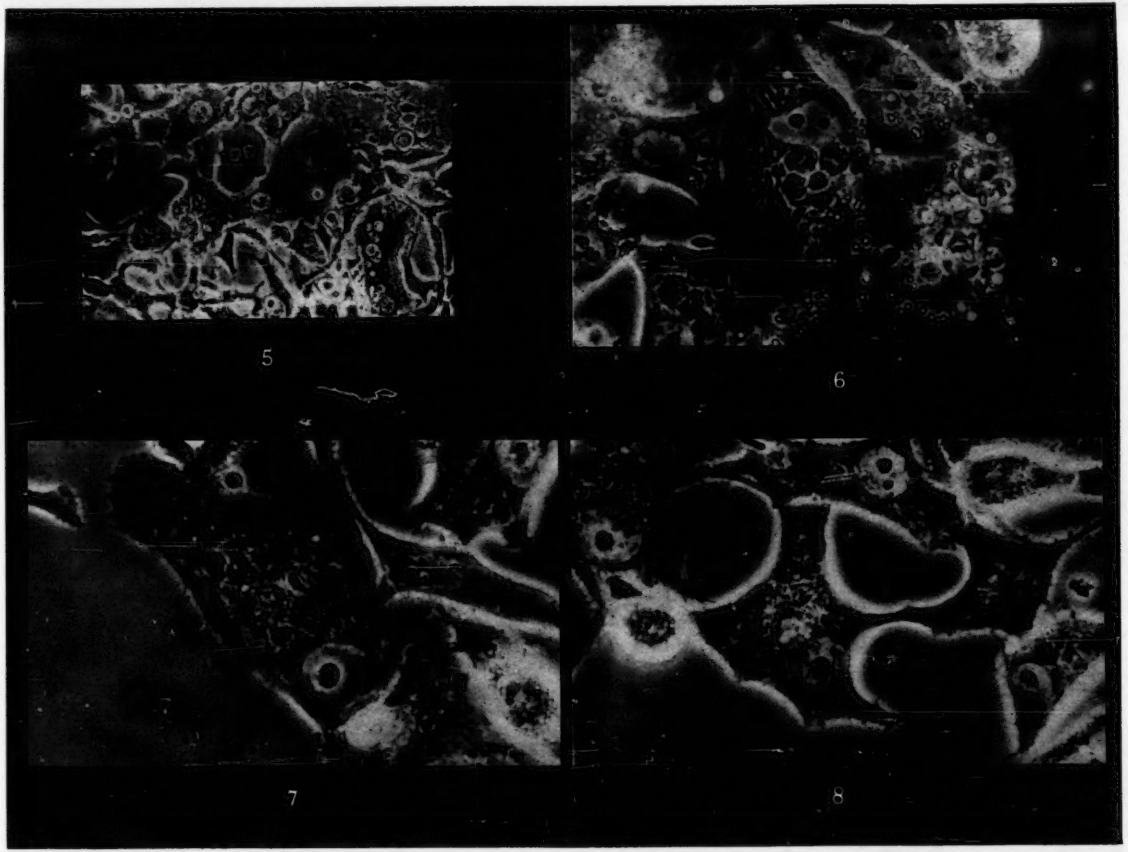

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As a first attempt to reveal on which cell activities parasite multiplication is dependent, the propagation of parasites in irradiated and that in non-irradiated cells was compared. It was found that such metabolic activities of the cytoplasm as may be directed from the nucleus and depend on its mitotic capability, did not influence the multiplication of the parasites.

SUMMARY

Toxoplasma parasites were irradiated with high energy gamma rays. It was found that irradiation inhibited the mitotic activity of the parasites. The antigenic properties of the parasites studied by the dye test were unaffected. Irradiated host cells without mitotic capacity supported multiplication of parasites just as did the non-irradiated cells.

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INTRACELLULAR KILLING OF *MICROCOCCUS LYSODEIKTICUS* BY MACROPHAGES AND POLYMORPHONUCLEAR LEUCOCYTES: A COMPARATIVE STUDY

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EXPERIMENTAL studies carried out on animals have shown that bacteria injected intravenously are rapidly cleared from the circulation by the reticuloendothelial system (RES) (Ørskov, Jensen and Kobayashi, 1928; Bennett and Beeson, 1954; Rogers, 1956; Halpern, 1959). Following this trapping of bacteria in the cells of the RES, destruction may follow (Pike and Mackenzie, 1940; Schneider and Zinder, 1956; Gorrill, 1956). Robertson and Van Sant (1939) were able to demonstrate phagocytosis and digestion of pneumococci by macrophages *in vitro* and studies using other bacteria have confirmed the ability of macrophages to phagocytose and destroy a variety of bacteria (Furness, 1958; Rowley, 1958; Jenkins and Benacerraf, 1960).

Recognition of the importance of intracellular killing in host defence mechanisms led to a search for cellular antimicrobial substances, although the granulocyte has been studied in more detail than the macrophage. These investigations resulted in the identification of several specific bactericidal agents whose properties have been fully reviewed by Skarnes and Watson (1957).

One bactericidal substance present in large amounts in the polymorphonuclear leucocyte is lysozyme (Fleming, 1922; Flanagan and Lionetti, 1955) but until recently granulocyte lysozyme has received little attention because, under the usual conditions of testing, its bactericidal range is restricted to saprophytic bacteria. Interest in lysozyme has been stimulated by the discovery that the mucopeptide that forms the lysozyme substrate is present in the bacterial cell walls of many pathogenic organisms including Gram negative species (Work, 1957; Weidel and Primosigh, 1958). A number of Gram negative bacteria are destroyed by egg-white lysozyme in the presence of a chelating agent (Repaske, 1956) and lysozyme is essential for the bactericidal and lytic action of serum on Gram negative organisms (Amano, Mariolsa, Seki and Kashiba, 1956; Muschel, Carey and Baron, 1959). These observations together with the work of Gladstone and Johnston (1955), which showed that under suitable cultural conditions *Bacillus anthracis* could be made sensitive to lysozyme, support the suggestion of Dubos (1954) and Hirsch (1956) that the intracellular environment may provide conditions under which lysozyme can destroy pathogenic bacteria.

In the present work *in vitro* systems of polymorphs and peritoneal macrophages have been used to make a quantitative study of phagocytosed bacteria. The role of lysozyme as an intracellular antimicrobial agent has been assessed by studying the differences between killing and lysis of lysozyme-sensitive bacteria and of their resistant variants. These experiments indicated that a lysozyme-like enzyme was

present not only in polymorphs but also in macrophages and therefore cell free extracts of both varieties of cell were prepared in order to carry out physicochemical and immunological examination of their lytic enzymes. Finally the action of these enzymes on bacterial suspensions was compared with that of egg-white lysozyme.

MATERIALS AND METHODS

Bacteria.—From a typical lysozyme-sensitive strain of *Micrococcus lysodeikticus*, resistant variants were obtained by serial subculture on nutrient agar containing increasing amounts of lysozyme. The most resistant of these variants, strain L250, was capable of growth on nutrient agar containing 4000 μg. lysozyme per ml. whereas the parent strain L2 failed to grow in the presence of 0·1 μg. per ml. of enzyme. A variant of intermediate resistance, strain R3, was able to grow on media containing 10 μg. per ml. lysozyme. The technique for the development of resistant strains of *M. lysodeikticus* has been described previously (Brumfitt, 1959).

Bacterial suspensions.—The various strains of *M. lysodeikticus* were grown on nutrient agar, harvested and suspended in Dulbecco A solution. The suspension was shaken vigorously to produce a suspension which was as uniform as possible and the opacity read using the Hilger absorptiometer with a neutral grey filter. By the use of a previously constructed curve relating optical density to bacterial count, the suspension was adjusted to contain the requisite number of bacteria. Precise enumeration of bacteria in this suspension was made subsequently.

Tissue Culture Methods

Medium

Medium number 199 (Morgan, Morton and Parker, 1950) free from antibiotics was used. Immediately before use 1 part of inactivated calf serum was added to 9 parts of 199 medium.

Macrophages

Rats were injected intraperitoneally with 5 ml. of sterile 1 per cent glycogen in distilled water. Four days later they were killed with chloroform and the peritoneal cavity of each washed out with 20 ml. of tissue culture medium containing 0·5 international units per ml. heparin. The washings contained approximately 10^6 cells per ml. of which 85–100 per cent were macrophages. The cell suspension was distributed in 2 ml. amounts into tubes which were stoppered after replacing the atmosphere with 5 per cent CO_2 in air. The tubes were then incubated at 37° for 24 hr. at an angle of 10° to the horizontal. At the end of this time the tubes were examined microscopically and any unsatisfactory cultures discarded.

Polymorphonuclear leucocytes

From the rat peritoneum.—If 1 per cent glycogen solution was injected in the way already described for macrophage production but the exudate washed out after only 4–5 hr., it was found that 40–60 per cent of the cells were polymorphs. A similar exudate was found 16 hr. after the injection of 3 per cent casein (Table).

TABLE.—*The Relation Between intraperitoneal injection and Lysozyme Activity of the Resulting Exudate*

Type of Exudate	Per cent Mononuclears	Per cent Polymorphs	Lysozyme μg./10^6 cells
Glycogen 4 days	100	0	0·43
,, 1 day	100	0	0·58
,, 4 days	91	9	1·0
,, 4 ,,	91	9	0·25
,, 5 ,,	86	14	0·1
Casein 16 hr.	61	39	0·25
Glycogen 4 hr.	56	44	0·14
,, 5 hr.	54	46	0·4
Casein 16 hr.	52	48	0·61
Glycogen 4 hr.	40	60	0·4

From human blood.—Human blood was drawn into a siliconed all glass syringe containing a trace of heparin. A solution of 6 per cent dextran in physiological saline (Intradex Glaxo) was added to the blood in the ratio 1 part dextran solution to 4 parts blood, well mixed and allowed to stand in a conical centrifuge tube for 40 min. Rouleau formation, induced by the dextran, resulted in rapid sedimentation of the red cells whilst the white cells remained in the supernatant plasma. The latter was pipetted off and the white cells deposited by centrifugation at 1000 r.p.m. for 5 min., the supernatant being removed and discarded. The white cells were resuspended in Dulbecco A solution to which 20 per cent serum from the same subject had been added so as to give a cell concentration of about 5×10^6 per ml., accurate counts being carried out subsequently.

Preparation of cell free extracts of macrophages and polymorphonuclear leucocytes

Peritoneal exudates containing either macrophages or polymorphonuclear leucocytes were prepared as described above and the cells deposited by centrifugation and resuspended in Dulbecco A solution. Preliminary experiments showed that if these cells were subjected to further washing an appreciable, but variable amount of cell lysis occurred. Fortunately it was also found that the supernatant from the initial peritoneal exudate contained no detectable lysozyme and accordingly it was considered that further washing of the cells was unnecessary.

A sample of the cell suspension was taken for total and differential cell counts (Table) and the remainder lysed by repeated freezing and thawing using a bath containing solid carbon dioxide and acetone. This lysate was centrifuged at 2000 r.p.m. for 10 min. to remove gross debris and the supernatant removed and stored at −20°.

Intracellular Destruction of M. Lysodeikticus *Following Phagocytosis*

Intracellular killing by macrophages

From each of a series of tubes containing cultures of macrophages the 2 ml. of tissue culture medium was removed and replaced by 2 ml. of similar medium containing approximately 2×10^6 viable bacteria. The atmosphere in the tubes was replaced by 5 per cent CO_2 in air, the tubes replaced in the 37° incubator and phagocytosis allowed to proceed. After 15 min. the supernatant fluid containing unphagocytosed bacteria was poured off and the macrophages washed gently but thoroughly with 3 changes of Dulbecco A solution at 37° in order to remove any organisms remaining unphagocytosed. Now 2 ml. of fresh tissue culture medium containing no bacteria was added to the tubes and the atmosphere again replaced by 5 per cent CO_2 in air. Two tubes were used to assess the maximal count of viable phagocytosed bacteria and this was taken to represent the zero reading. The remainder of the tubes were returned to the incubator and further pairs of tubes were removed after 15, 30, 60 and 90 min. and viable counts carried out. In this way it was possible to plot curves relating intracellular survival of bacteria to time. Counting of viable phagocytosed bacteria was carried out by the following technique. The tissue culture medium was poured off and replaced by 2 ml. of nutrient broth. The sides of the tube were now rubbed with a sterile rubber capped glass rod and this procedure (Furness, 1958) in the presence of nutrient broth, disintegrated the macrophages and released any unkilled bacteria. By the use of standard dilution techniques followed by inoculation on to nutrient agar plates, it was possible to count the number of viable intracellular bacteria. To guard against errors due to unphagocytosed bacteria adhering to the walls of the tubes or the macrophages, the following control experiment was carried out. Bacterial suspension was added to 2 tubes containing macrophages but instead of allowing 15 min. for phagocytosis to occur, the bacterial suspension was poured off immediately and the macrophages washed. The full procedure for counting viable phagocytosed bacteria was then performed as described above. Since the time of contact between bacterial suspension and macrophages was very brief, little or no phagocytosis could have occurred and the bacterial count obtained could be taken to represent bacteria adhering to the outside of the macrophages and walls of the tube. The count obtained was always small and is referred to in Fig. 1 as minus 15 min.; in contrast to the zero reading which is the number of viable intracellular bacteria after 15 min. had been allowed for phagocytosis.

Intracellular killing by polymorphonuclear leucocytes

The technique used was based upon that of Cohn and Morse (1959). Bacterial and leucocyte suspensions were agitated so as to make them as homogeneous as possible and warmed to 37°.

Exactly 1 ml. amounts of the 2 suspensions were mixed in 3 × $\frac{3}{8}$ in. siliconed tubes and incubated for 7 min., continuous mixing being used to maintain a homogeneous suspension. The tube was then centrifuged at 450 r.p.m. for 4 min. to sediment the leucocytes together with phagocytosed bacteria. Unphagocytosed bacteria remain in the supernatant and were pipetted off, the leucocytes resuspended in 2 ml. Dulbecco A solution and the centrifugation repeated. The supernatant was again removed and the deposit resuspended in 1 ml. Dulbecco A solution. After thorough mixing, a sample of 0·1 ml. was removed and used to prepare suitable dilutions in nutrient broth. Viable counts were now carried out using the technique described for the macrophages. As a control an experiment was carried out in which bacteria and leucocytes were mixed and immediately separated again by centrifugation, the deposit washed and a count performed.

Intracellular lysis by macrophages

Preliminary experiments with bacterial suspensions and egg white lysozyme showed a close correlation between the speed of killing and the speed of lysis of *M. lysodeikticus*. This finding is not surprising when it is recalled that the lysozyme substrate is situated in the cell wall and the action of the enzyme causes weakening and consequently rupture of the cell wall followed by both lysis and death of the bacterium.

Since stained preparations of macrophages showed that progressive lysis of phagocytosed *M. lysodeikticus* took place, it was decided to study this lysis on a quantitative basis and to compare the results with the killing curves previously described.

The preparatory stages of this experiment are very similar to those described for viable counting of phagocytosed bacteria but coverslips were included in the tubes so that macrophages coated their surface. Instead of destroying the macrophages and carrying out viable counts, cover-slips were removed at intervals, fixed with methyl alcohol, stained with 1 per cent aqueous methylene blue or Leishman's stain and mounted on glass slides.

The stained preparations were examined microscopically and the proportion of cells containing intact bacteria and the proportion containing bacterial debris recorded. The usual technique of counting was to examine 1000 macrophages in two different preparations and to calculate the average results from the two counts.

Intracellular lysis by polymorphonuclear leucocytes

The fundamental difference in technique from that of assessment of intracellular killing by polymorphs described above was that instead of performing viable counts, samples of suspension were spread on grease free microscope slides and then fixed, stained and counted in the way described for the macrophage preparations.

RESULTS

Intracellular Destruction of M. lysodeikticus *Following Phagocytosis*

Intracellular killing by macrophages

Fig. 1 summarizes the results obtained. With strain L2 substantial uptake of cocci occurred during the 15 min. allowed for phagocytosis (−15 min. to 0 in Fig. 1) and subsequently rapid intracellular killing followed so that after 90 min. few viable bacteria remained. With strain L250 the initial uptake by the macrophages was similar to that with L2 but in contrast little killing took place. It is known that resistance of strain L250 is due to acetylation of cell wall hydroxyl groups and that deacetylation which can be brought about by exposure to alkali, restores susceptibility to lysis by egg-white lysozyme (Brumfitt, Wardlaw and Park, 1958). When L250 was deacetylated with glycine buffer at pH 11, lysozyme sensitivity was restored without loss of viability. The susceptibility of L250 to intracellular killing following deacetylation at pH 11 is shown by the interrupted line in Fig. 1 ; this treatment restored the susceptibility of the organism to intracellular killing.

Intracellular killing by polymorphonuclear leucocytes

The results of these experiments are shown in Fig. 2 and 3. During the 7 minute period allowed for phagocytosis (−7 to 0 in Figs. 2 and 3), good uptake occurred with strains L2, R3 and L250. Subsequently, strain L2 was rapidly destroyed so

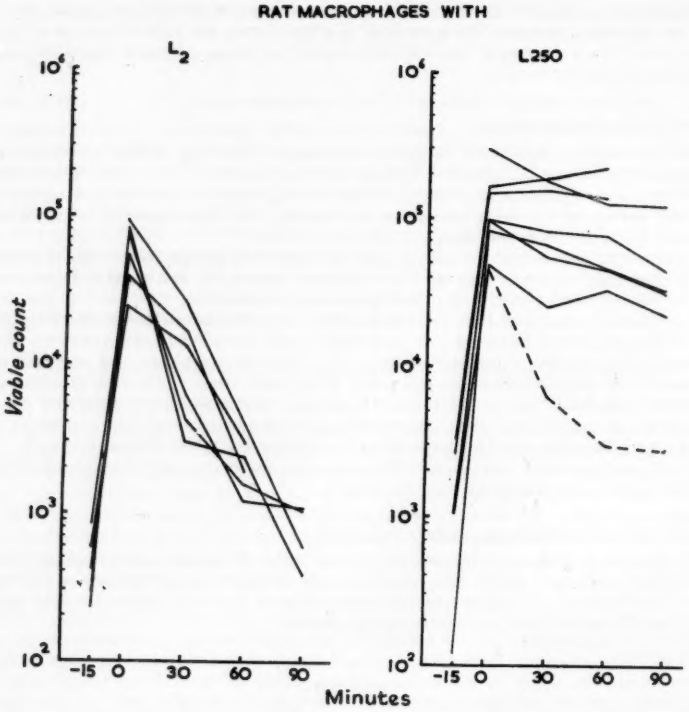


FIG. 1.—Intracellular killing of *M. lysodeikticus* strains L2 and L250 by rat macrophages. A period of 15 min. (−15 to 0 min.) was allowed for uptake of bacteria and intracellular destruction was estimated from time zero. The interrupted line in the right hand graph shows the result of an experiment using L250 after deacetylation.

that after 60 min. few viable intracellular bacteria remained. By comparison the partially lysozyme-resistant strain R3 was less rapidly destroyed and substantial numbers of intracellular bacteria remained viable after 1 hr. (Fig. 2), whilst with L250, no killing was evident during the time of the experiment. However, deacetylation of L250 with pH 11 glycine buffer caused it to become highly susceptible to intracellular destruction (Fig. 3).

Intracellular lysis by macrophages and polymorphonuclear leucocytes

Phagocytosis of strain L2 by both macrophages and granulocytes was followed by rapid lysis which resulted in the disappearance of intact bacteria and the

appearance of bacterial debris. Experiments with strain L250 showed this organism to be almost entirely resistant to lysis and consequently very little bacterial debris was found (Fig. 4 and 5).

An example of the rapid intracellular lysis with strain L2 is seen in Fig. 6*a* and *b* which show stained macrophage preparations. Fig. 6*b* shows intact bacteria

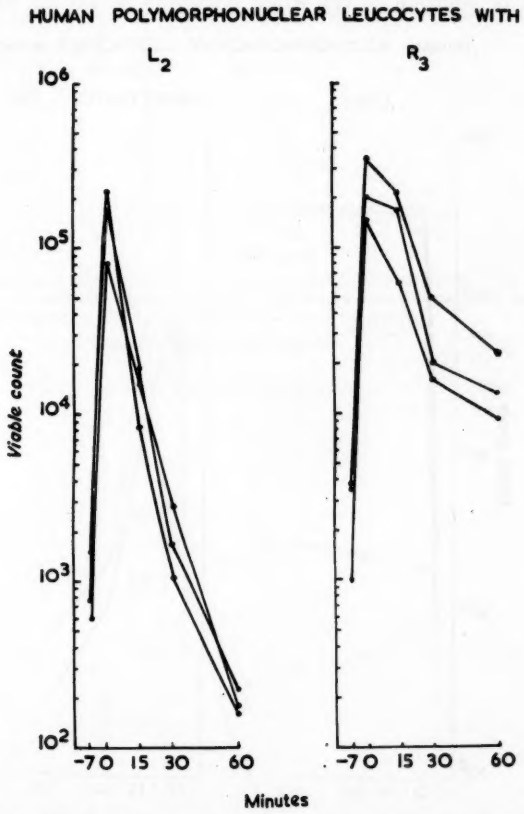


FIG. 2.—Intracellular killing by human polymorphs of a lysozyme sensitive strain of *M. lysodeikticus* (L2) and a semi lysozyme-resistant strain (R3). A period of 7 min. was allowed for phagocytosis (−7 to 0 min.).

within macrophages immediately after being phagocytosed. Thirty minutes later L2 has been totally lysed and only cytoplasmic vacuoles remain (Fig. 6*a*).

A low power view of polymorphs which have phagocytosed L250 (Fig. 7) shows that a preparation made immediately after phagocytosis (Fig. 7*a*) and another 1 hr. later (7b) are indistinguishable. This illustrates the resistance of L250 to intracellular lysis.

Identification of Bactericidal and Lytic Substances in Cell Free Extracts of Macrophages and Polymorphonuclear Leucocytes and Their Comparison with Egg-white Lysozyme

Lysis of strains of M. lysodeikticus

Lysis curves were constructed by adding 0·5 ml. of the appropriate dilution of cell free extract or solution of egg-white lysozyme to 4·5 ml. of a standardized

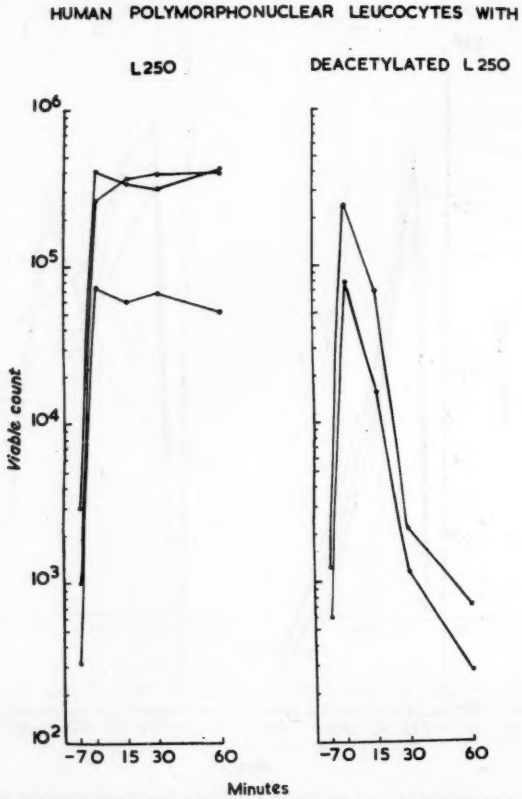


FIG. 3.—The effect of deacetylation of a lysozyme-resistant *M. lysodeikticus* strain L250 on intracellular survival after phagocytosis by polymorphs.

suspension of *M. lysodeikticus* in 0·15 M phosphate buffer at pH 6·4. Reduction in turbidity was estimated with a Spekker absorptiometer using neutral grey filters. In addition, quantitative estimation of lysozyme was made by a modification of the method of Hook, Carey and Muschel (1960) after a reaction time of 10 min. at 37°.

The results obtained with the sensitive strain of *M. lysodeikticus* (L2) with macrophage and polymorph extracts and their comparison with egg white lyso-

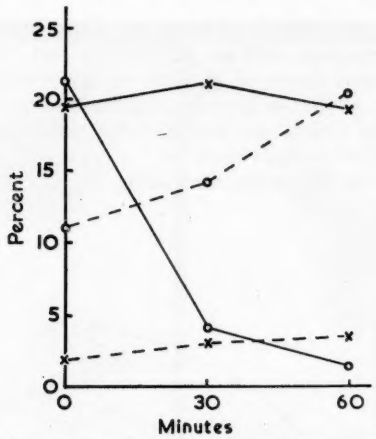


FIG. 4.—Bacterial lysis within rat macrophages as judged by staining. The solid lines show the percentage of cells containing bacteria. The rapid disappearance of intact bacteria of strain L2 is accompanied by the appearance of bacterial debris.

Percentage cells with intact bacteria ×———× L250
○———○ L2
Percentage cells with bacterial debris ×- - - -× L250
○- - - -○ L2

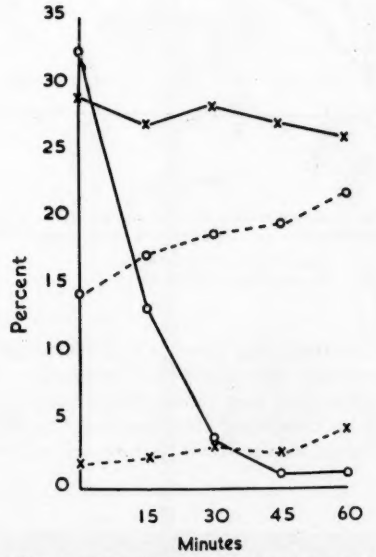


FIG. 5.—Bacterial lysis within human polymorphonuclear leucocytes as judged by staining.

Percentage cells with intact bacteria ×———× L250
○———○ L2
Percentage cells with bacterial debris ×- - - -× L250
○- - - -○ L2

zyme are shown in Fig. 8 and 9. Fig. 8 shows the lysis curve produced on lysozyme-sensitive L2 by a macrophage extract and by 0·1 and 0·5 μg. per ml. egg white lysozyme. The slope and shape of the curves are similar and the macrophage extract diluted 1 in 20 is almost superimposed on the curve produced by 0·1 μg. per ml. lysozyme. From this it can be concluded that the undiluted macrophage extract has a lytic activity equivalent to 2 μg. per ml. of egg white lysozyme. Fig. 9 shows a similar set of curves comparing polymorph extract and egg-white

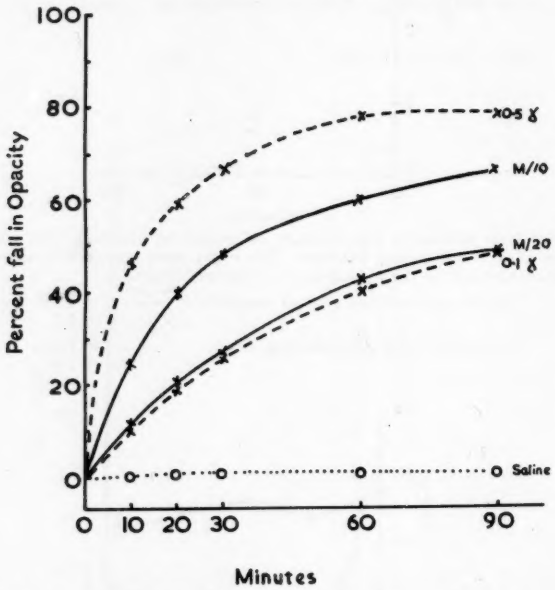


FIG. 8.—Curves showing lysis of strain L2 ; comparing the effect of egg white lysozyme of known concentration with dilutions of macrophage extract.

Egg white lysozyme ×- - - -×
Macrophage lysozyme ×————×

lysozyme. Here it is apparent that there is a difference in the slope of the curves between polymorph extract and egg-white lysozyme. Despite this difference, evidence that both polymorph and macrophage extracts behave like egg white lysozyme was obtained by observing the effects of the extracts upon the resistant variant of *M. lysodeikticus*, strain L250, before and after deacetylation. Fig. 10

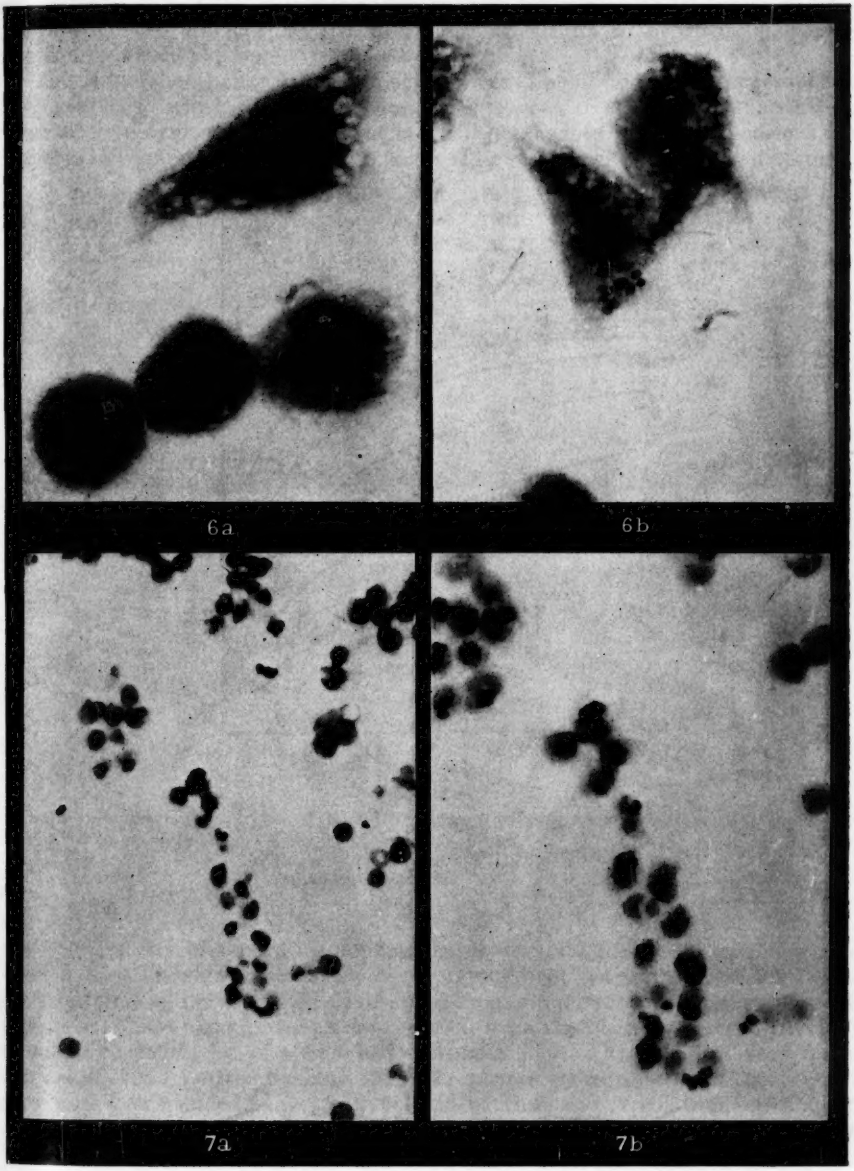
EXPLANATION OF PLATE

FIG. 6*b*.—Photograph immediately after phagocytosis of L2 by macrophages showing darkly staining intact bacteria.

FIG. 6*a*.—After only 30 min. no bacteria remain and vacuoles can be seen in the macrophage cytoplasm.

FIG. 7*a*.—Photograph immediately after phagocytosis by polymorphs of strain L250.

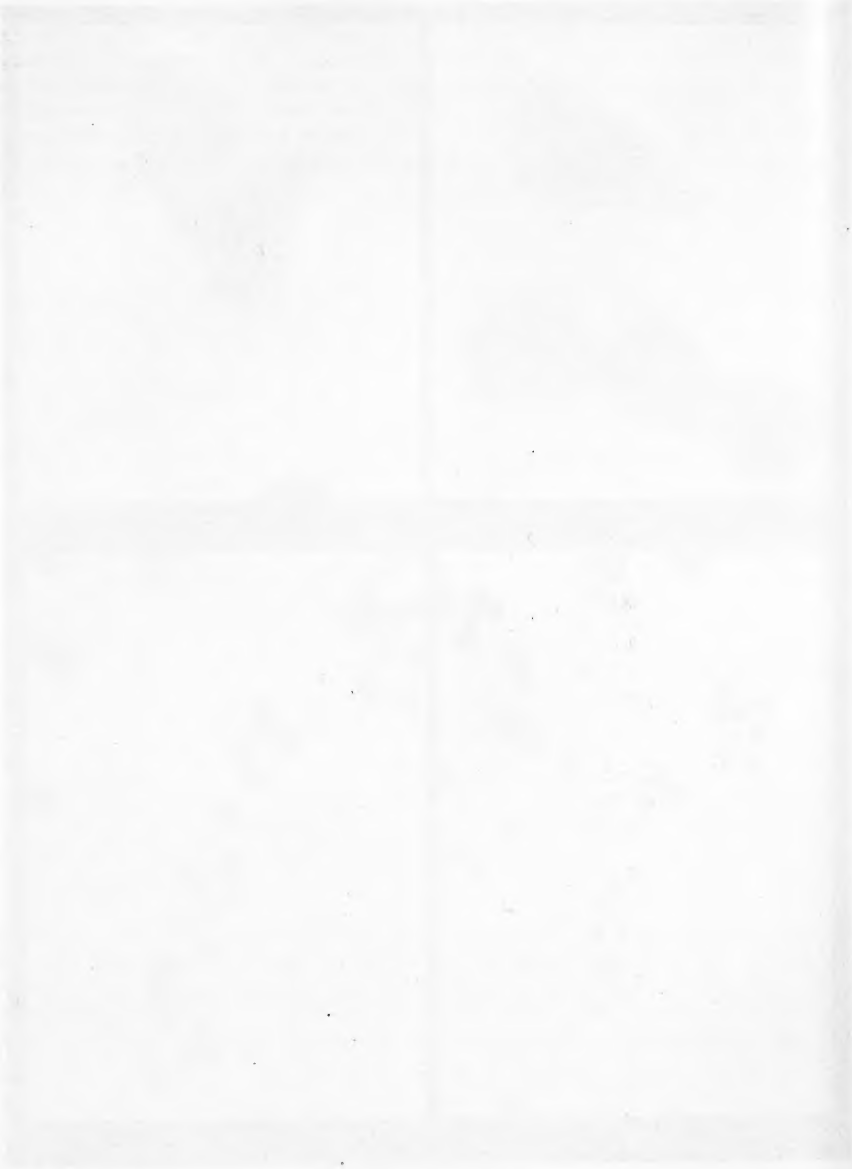
FIG. 7*b*.—The same preparation 1 hr. later showing complete resistance to intracellular lysis.



6a 6b

7a 7b

Brumfitt and Glynn.



shows that strain L250 is insusceptible to lysis by egg white lysozyme and by macrophage and polymorph extracts whereas sensitivity to all three enzymes is restored by deacetylation.

The Table shows that good lytic activity could be obtained with macrophage preparations in which no polymorphonuclear leucocytes could be demonstrated. Even where polymorphs were present in small numbers in the cell suspensions the lytic activity could not be accounted for by their presence. However, to determine

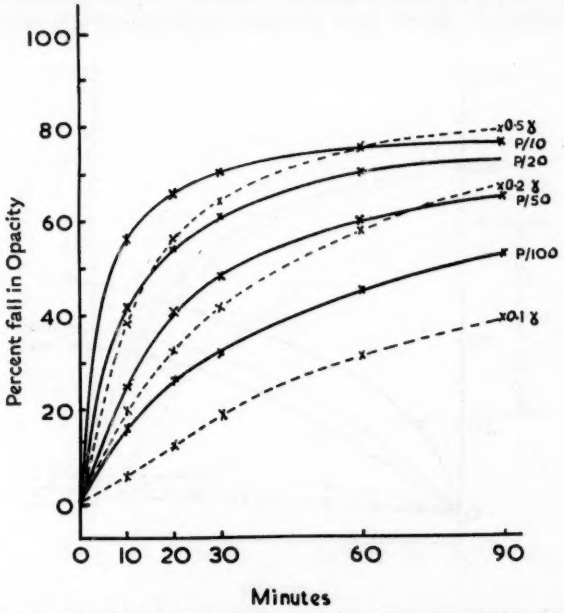


FIG. 9.—Curves comparing lysis of strain L2 by egg white lysozyme and dilutions of polymorph extract.

Egg white lysozyme ×- - - -×
Polymorph lysozyme ×———×

the lytic activity of a pure macrophage suspension, rat peritoneal exudates produced 4 days after injection of glycogen were grown in tissue culture for 48 hr. by which time all polymorphs had disappeared and a pure macrophage population remained. These macrophages were removed from the glass by versene (EDTA) at a final concentration of 1/5000 in Dulbecco A solution (Lwoff, Dulbecco, Vogt and Lwoff, 1955). After washing the macrophages twice to remove the versene they were resuspended in a small volume of Dulbecco A solution, an aliquot removed for counting, and the remainder lysed by freezing and thawing. The lysozyme activity in the macrophage extract was measured and knowing the number of macrophages used to prepare the extract, the equivalent lysozyme concentration of each cell was calculated. From duplicate estimations on each of two batches of macrophages the lysozyme concentration was found to be within the range

0·82–0·87 $\times$ 10^{-6} μg. per cell. If macrophage lysozyme is assumed to have the same molecular weight as that of egg white lysozyme, there would be about 35 $\times$ 10^6 molecules of lysozyme per macrophage.

Physico-chemical properties of egg-white lysozyme and macrophage and polymorph enzyme

The number of tests possible was restricted by the small quantities of macrophage and polymorph extract available. The lytic enzymes were relatively heat stable in acid solution. At 37° they showed a fairly broad pH optimum for lysis,

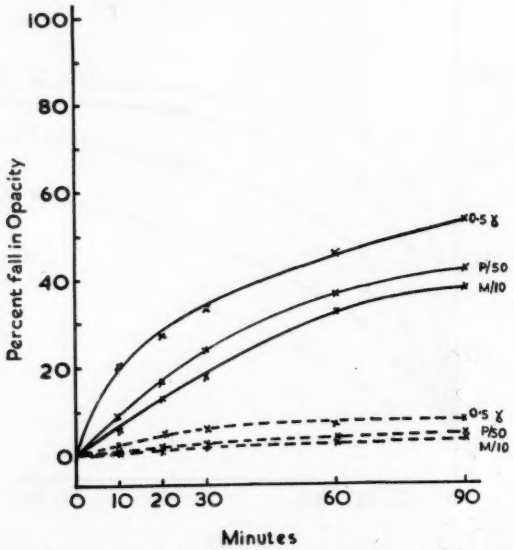


FIG. 10.—Lysis curves showing the effect of cell extracts and egg white lysozyme on strain L250 before and after deacetylation.

optimal between 6 and 6·5 with a very rapid decline above pH 7·0 and below 5·5. All had a small molecular weight since they escaped from a Visking membrane at about the same rate. After 18 hr. dialysis approximately one-third of the activity was lost. The molecular weight of egg white lysozyme is 14,600 (Fevold, 1951) but Fraenkel-Conrat (1950) has pointed out that diffusibility depends also on molecular shape and charge. Thus lima bean trypsin inhibitor with a molecular weight of 10 000 is completely retained by a dialysing membrane. It is reasonable to suppose therefore that egg white polymorph and macrophage lysozymes are of similar molecular size shape and charge, However the cell lysozymes may be in bound forms in the intact cell.

Immunological properties of egg white, macrophage and polymorph lysozyme

Egg white lysozyme is antigenic in rabbits (Roberts, 1937 ; Smolens and Charney, 1947 ; Wetter and Deutsch, 1951) and antibody was prepared by intramus-

cular injection together with Freund's adjuvant. Antibody so produced can be detected by agglutination of tanned red cells coated with lysozyme or by direct precipitation tests. If egg white lysozyme is allowed to react with its antibody, its lytic action on suspensions of L2 is inhibited, even though precipitation of the antigen-antibody complex has not occurred. Fig. 11 shows the results of an experiment in which the effect upon lysis of adding varying amounts of antibody to 0·5 μg./ml. egg-white lysozyme was investigated.

It is seen that with increasing amounts of antibody there was increasing inhibition of lysis. When the same antibody was added to macrophage or poly-

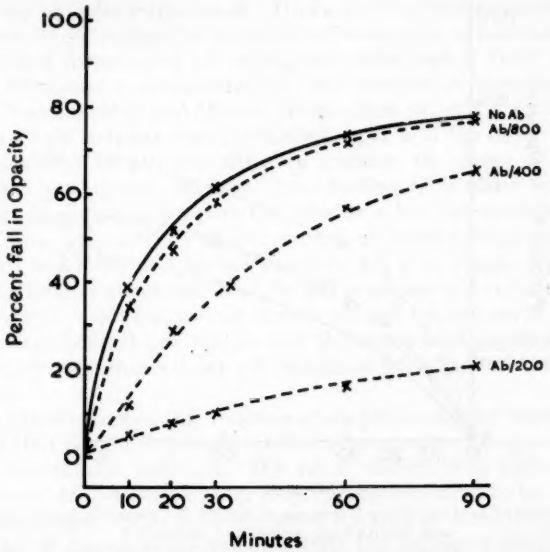


FIG. 11.—Lysis curves of strain L2. The inhibitory effect on egg white lysozyme of combination with specific antibody. As the quantity of antibody is increased, the activity of the lysozyme is progressively inhibited.

morph lysozyme no inhibition of their ability to lyse suspensions of L2 occurred nor could any combination between these lysozymes and egg white lysozyme antibody be demonstrated to have taken place.

DISCUSSION

The present experiments have shown that a lysozyme-sensitive strain of *M. lysodeikticus* (L2) was destroyed within both human polymorphs and rat macrophages following phagocytosis. It was also found that within these cells killing and lysis occur at the same speed which is exactly what happens when egg white lysozyme is added to a suspension of L2.

On the other hand a lysozyme-resistant variant (strain L250) was able to survive within these cells following phagocytosis. It has previously been shown

that strain L250 differs genotypically from strain L2 in its ability to acetylate cell wall hydroxyl groups (Brumfitt, Wardlaw and Park, 1958). Furthermore, by using a glycine buffer at pH 11 deacetylation occurred and the bacteria became sensitive to egg white lysozyme once again without loss of viability. It was found that following deacetylation, intracellular destruction of strain L250 was brought about by both polymorphs and macrophages. It has also been demonstrated that cell free extracts of both rat macrophages and polymorphs contain a substantial amount of enzyme which has lytic and other physico-chemical properties very close to those of egg white lysozyme. From the evidence obtained, it is

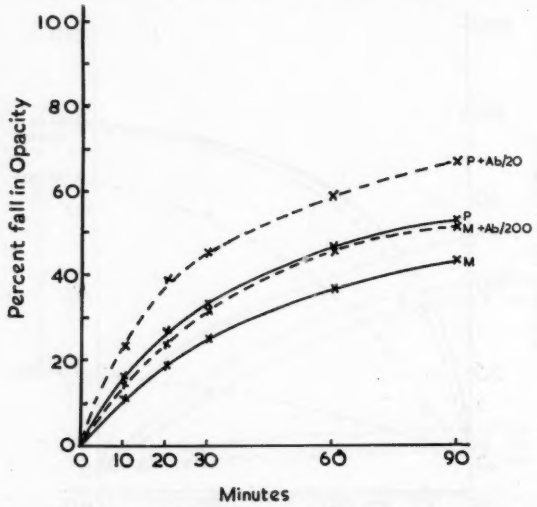


FIG. 12.—The failure of anti egg white lysozyme to inhibit the lysozyme like activity of polymorph and macrophage extracts on strain L2.

concluded that lysozyme is present not only in human and rat granulocytes but also in rat macrophages. The presence of lysozyme within polymorphs has long been recognised (Fleming, 1922) but, in contrast, the presence of lysozyme in peritoneal macrophages has actually been denied (Hirsch, 1960 ; Myrvik, 1960). The latter statement may be explained by variables such as species differences and method of producing the cellular exudate. We found that quantitatively the rat peritoneal macrophage contains approximately the same amount of lysozyme as the rat polymorph.

A rabbit antibody to egg-white lysozyme combined with and inhibited the action of egg white lysozyme, but failed to combine with or inhibit polymorph or macrophage lysozyme. Since all three lysozymes act on the same substrate, their active centres are probably similar. It seems likely, therefore, that the antibody fixes itself to some other part of the molecule but prevents the action of egg white lysozyme by steric hindrance and that it is these other parts of the egg white lysozyme molecule which differ from those of macrophage and polymorph lysozymes.

It has already been shown by Jollés and Fromageot (Jollés, 1959) in their analyses of the various lysozymes, all of which are small molecular weight basic proteins with a high arginine content, that they differ slightly in amino acid composition. These workers studied lysozyme from a dog's spleen and two others from a dog's kidney, one of which was certainly different from the splenic lysozyme. All three differed from two lysozymes from rabbit spleen and egg-white lysozyme. It is, therefore, not surprising that differences between egg white and cellular lysozymes can be detected.

The role of lysozyme as a tissue antimicrobial factor is still uncertain. The limited antibacterial spectrum led earlier workers (*e.g.* Thompson, 1941) to conclude that it was of little importance. However, the combination with chelating agents (Repaske, 1956) makes lysozyme effective against a number of Gram negative pathogens and Amano and his colleagues (1954*a* and *b*, 1955) have shown the lytic effect of lysozyme is accelerated in the presence of specific antiserum and complement. Dubos (1954) and Hirsch (1956) drew attention to the observations of Rous (1925) which indicate that intracellular pH is of the order 3–5 and suggest that the environment within the cell may increase the range of effectiveness of lysozyme against pathogenic bacteria. Our finding of marked intracellular lysis of *M. lysodeikticus* does not support the idea of a low intracellular pH, since we found that *in vitro* lytic activity of polymorph and macrophage lysozyme is inhibited at acid pH and minimal lysis occurs below pH 5·0. The rapid lysis which was observed intracellularly suggested that the pH was near the optimum for lysozyme action. However, it is not impossible that in the cell variations of pH occur in the vicinity of a phagocytosed particle to suit the needs of a particular enzyme and conclusions concerning intracellular pH based on such limited evidence would be unwise.

Mackaness (1960) studied inactivation of staphylococci by rabbit macrophages and concluded that the antibacterial mechanisms of macrophages and polymorphs might be fundamentally different. We have shown that killing of lysozyme-sensitive strains of *M. lysodeikticus* by polymorphs appeared to be more rapid than killing by macrophages but in both instances the major bactericidal substance was lysozyme. It is, of course, quite possible that the killing of staphylococci within the cell depends upon unrelated systems.

Furness (1958) studied the fate of avirulent and virulent strains of *Salm. typhimurium* following phagocytosis by mouse macrophages and altered monkey kidney cells. He found that the avirulent strain was destroyed whereas the virulent strain multiplied within the phagocytic cell and ultimately caused its lysis. There was no evidence that virulence was determined by the bacterium being able to resist phagocytosis.

Because of its slow rate of growth no information was obtained about intracellular multiplication of L250 during the limited time of the present experiments and no damage to the phagocytic cell was observed. Strains L2 and L250 were phagocytosed equally well in the absence of specific antibody or complement.

The ability to survive within the macrophage or polymorph does not appear to be associated with any enhancement of virulence of strain L250 as judged by a number of pathogenicity tests including intracerebral inoculation carried out on mice. It is known that the genetic changes necessary for development of lysozyme resistance by *M. lysodeikticus* greatly reduce the growth rate (Brumfitt, 1959) and this, together with other metabolic changes, may account for the lack of virulence.

It may therefore be concluded that both macrophages and polymorphonuclear leucocytes contain lysozymes which resemble one another quantitatively and qualitatively and which are similar to egg white lysozyme. From the results obtained it is not possible to say whether intracellular lysozyme plays any part in the destruction of pathogenic bacteria following phagocytosis, though the evidence available suggests that this is a distinct possibility and worthy of further investigation.

SUMMARY

Lysozyme has been demonstrated in rat peritoneal macrophages and its presence in human and rat polymorphonuclear leucocytes confirmed.

In vitro experiments with macrophages and polymorphonuclear leucocytes showed that, in the presence of calf serum, egg white lysozyme sensitive (L2) and egg white lysozyme resistant strains (L250 and R3) of *M. lysodeikticus* were phagocytosed equally well. The subsequent fate of these phagocytosed bacteria was followed by making serial viable counts of intracellular bacteria and also by quantitative assessment of bacterial lysis in stained preparations. These two parallel methods of assessment, made over a period of $1\frac{1}{2}$ hr. from the time of phagocytosis, showed rapid killing and lysis of the lysozyme sensitive strain L2. On the other hand the highly lysozyme resistant strain L250 showed neither lysis nor loss of viability. By chemical treatment of strain L250 it was possible to restore its lysozyme-sensitivity without loss of viability and organisms treated in this way were susceptible to both intracellular lysis and killing. A strain of *M. lysodeikticus* moderately resistant to egg white lysozyme (R3) showed a rate of intracellular destruction intermediate between L2 and L250. In all experiments there was an exact correlation between intracellular lysis and killing. To further investigate the destruction of the various *M. lysodeikticus*, strains by macrophages and polymorphs cell free extracts of these cells were prepared. It was found that these extracts lysed the egg white lysozyme sensitive strain, L2, but not its resistant variant L250. The form of the lysis curve, dialysis properties, pH and temperature dependence were similar with the cell extracts and egg white lysozyme.

On the basis of the phagocytosis experiments and those with cell extracts, it was concluded that polymorphs and macrophages both contain lysozyme, and that this enzyme plays a major role in killing phagocytosed *M. lysodeikticus*.

The possible significance of lysozyme in the intracellular killing of other organisms is discussed.

It was found possible to distinguish cell and egg white lysozyme immunologically. An antibody prepared against egg white lysozyme inhibited the latter's lytic activity but had no effect on polymorph or macrophage lysozyme.

Although the resistant strain L250 was able to survive within polymorphs, its virulence as judged by conventional pathogenicity tests was no greater than that of the sensitive parent strain L2. This lack of virulence may be due in part to the great slowing of the growth rate which is found in highly lysozyme resistant mutants of *M. lysodeikticus*.

We are indebted to Dr. G. Furness of the Lister Institute for helpful discussion and practical advice in the design of the macrophage culture experiments.

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NEWCASTLE DISEASE VIRUS (NDV) IN TISSUE CULTURES OF RABBIT CORNEAL ENDOTHELIUM: VIRAL MULTIPLICATION AND CYTOPATHOGENICITY*

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THE introduction of large amounts of certain viral agents into the anterior chamber of the rabbit eye leads to the development of corneal opacity (Evans and Rickard, 1945; Evans and Bolin, 1946; Bolin, Anderson and Leymaster, 1950; Wilcox, Wood, Oh, Everett and Evans, 1958) and characteristic microscopic lesions of the corneal endothelium (Wilcox *et al.*, 1958; Oh and Evans, 1960) without a concomitant rise in virus titre.

"Toxicity" of myxoviruses in some tissue culture systems (Henle, Girardi and Henle, 1955) and in mouse brain (Schlesinger, 1950) appears to be associated with abortive infection. A similar mechanism may be involved in the corneal toxicity. It was also suggested that so-called toxicity of influenza and Newcastle disease virus (NDV) for the rabbit cornea might be due to an ordinary infection, not recognized as such because non-specific viral inhibitory substances in aqueous humour masked the multiplication of virus in the corneal endothelium (Wilcox *et al.*, 1958). With these possibilities in mind, it seemed worthwhile to study the interaction of NDV with corneal endothelial cells grown in tissue culture although many characteristics of *in vitro* infection with a virus are not directly applicable to an understanding of the pathology and pathogenesis of the lesions produced *in vivo*.

The present investigation was carried out with the following objectives in mind: to characterize cytopathic effects of NDV in tissue cultures of rabbit corneal endothelium, to determine whether the tissue cultures of rabbit corneal endothelium will support viral multiplication, and to determine the intracellular sites of synthesis of virus or viral antigen in the tissue cultures.

MATERIALS AND METHODS

Cultivation of rabbit corneal endothelium

Cultures were made according to the method described by Stocker, Eiring, Georgiade and Georgiade (1958). Rabbits weighing approximately 8 lb. were used as the source of cells. Under the dissecting microscope Descemet's membrane with the endothelial cells was peeled from a piece of the cornea. This was transferred to a 6 × 22 mm. cover slip which had been previously coated with rabbit plasma. The plasma was clotted by the addition of 1–2 drops of chick embryo extract. This preparation was transferred into a Leighton tube containing 1 ml. of culture medium consisting of 30 per cent heat-inactivated normal rabbit serum, 70 per cent medium 199 (Difco, U.S.A.), 100 units of penicillin and 100 μg. of streptomycin.

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The tube was plugged with a rubber stopper and incubated at 37°. Ten-day-old tissue cultures were used throughout the experiments. The medium was changed 2 days before the culture was used.

Virus

The L-Kan 1948 strain of NDV, a heat-resistant strain (Hanson, Upton, Brandly and Winslow, 1949) obtained from Dr. H. Rubin, was used. It was cultivated in 10-day-old embryonated hens' eggs by inoculating 10^4 plaque-forming units (PFU) of stock virus in a volume of 0·1 ml. into the allantoic cavity. Allantoic fluid harvested 48 hr. following inoculation was used in the experiments. NDV infectivity was determined by using the plaque count technique on a monolayer of cells derived from chick embryo (Dulbecco, 1952). Haemagglutinin titre was determined by the method described by Salk (1944).

Anti-NDV rabbit serum

Specific anti-NDV serum was prepared from rabbits given repeated injections of NDV which was partially purified by adsorption-elution with chick red blood cells and suspended in phosphate-buffered saline (PBS). The antiserum had 1200 HI units per ml. For studies utilizing the indirect fluorescent antibody technique, the specific antiserum was absorbed twice with chick embryo powder. The titre of the absorbed antiserum was 1000 HI units per ml. The chick embryo powder was prepared according to the method described by Coons, Leduc and Connolly (1955).

Fluorescent staining procedure

The indirect method described by Weller and Coons (1954) was used. A few drops of a 1/10 dilution of the anti-NDV rabbit serum were placed on the acetone-fixed cells and left in a humidity chamber at room temperature for 30 min. The cover slip with the cells was washed in 4 changes of PBS over a period of 10 min., covered with 2 drops of a 1/10 dilution of fluorescein-labelled anti-rabbit-γ-globulin goat serum (Microbiological Associates, U.S.A.) and incubated for 30 min. at room temperature. Then the preparation was washed with 4 changes of PBS during 10 min., air-dried and mounted on a slide with a drop of buffered glycerol. It was examined and photographed in a dark field using a Leitz Ortholux-UAM microscope with a 200 watt mercury vapour lamp as an ultraviolet light source.

Staining of tissue cultures of corneal endothelium for histological studies

Tissue cultures were fixed with Bouin's solution and stained with Harris haematoxylin and 1 per cent alcoholic eosin according to the standard staining procedure outlined by Lillie (1954).

RESULTS

Morphology of normal rabbit corneal endothelium in tissue culture

The endothelial cells commenced migration and proliferation in 1–2 days. A large confluent sheet of cells was noted after 10 days and had an average of 5×10^3 cells. The surface of these cells appeared to be free of the plasma clot which apparently dissolved during cell growth leaving the cell mass adherent to glass. Ten-day-old cultures of normal corneal endothelium consisted predominantly of polygonal cells, although a few spindle type cells were usually present at the periphery of the endothelial sheet.

In cultures stained with haematoxylin and eosin, each cell contained one or two round or oval nuclei with distinct round nucleoli (Fig. 1). Perinuclear cytoplasm was granular and eosinophilic. It was surrounded by faintly stained, homogeneous cytoplasm which outlined the border of the cell. Occasional mitotic figures were present in the sheet of cells (Fig. 1).

Cytopathic effects produced by NDV in tissue cultures of rabbit corneal endothelium

When tissue cultures of corneal endothelium were infected with 3×10^8 PFU of NDV in 1 ml. of the culture medium, progressive morphological changes were observed in the endothelial cells. The polygonal cells became spindle shaped or possessed several thread-like processes 24 hr. after virus inoculation (Fig. 2). The nuclei of these cells appeared to be normal. The cells were almost completely degenerated and only cell debris remained 48 hr. after inoculation. Tissue cultures inoculated with lesser amounts of virus, such as 10^6PFU, also showed the degenerative changes but these developed more slowly than with a large inoculum of virus. Tissue cultures inoculated with a mixture of virus and specific anti-NDV serum remained normal.

Besides the above degenerative changes, 2 characteristic cytopathic changes were observed. They were production of multinucleated cells, and formation of eosinophilic intracytoplasmic inclusions readily seen in cells fixed with Bouin's solution and stained with haematoxylin and eosin. These changes were not observed in normal tissue cultures of corneal endothelium incubated for various lengths of time, in cells inoculated with a lesser amount of virus, such as 10^6 PFU, or in cells inoculated with a mixture of NDV and specific antiserum. These changes appear to have been induced by the virus.

Multinucleated cells.—Multinucleated cells were found in occasional cultures infected with a large amount of the virus. The number of nuclei in some cells reached over 50. These nuclei were located in a random fashion in the cytoplasm or packed in a ring form surrounding a markedly granular eosinophilic cytoplasm (Fig. 3). Nucleoli were irregular in shape and larger than those of normal cells. The multinucleated cells began to appear 18 hr. after the virus inoculation. The cytoplasm of several corneal endothelial cells appeared to have fused to form a large common cytoplasm which was less granular than that of a normal cell. At 24 hr. after the initial infection, the nuclei of these fused cells appeared to aggregate in a somewhat contracted cytoplasmic mass, forming a readily recognizable multinucleated cell.

Eosinophilic intracytoplasmic inclusions.—Inclusions were well defined, irregularly shaped, eosinophilic and homogeneous in appearance. They were located in the cytoplasm, surrounded in some instances by a clear halo (Fig. 4). Inclusions began to appear in a few cells 24 hr. after initial exposure to virus, at a time when cellular degeneration was only moderate. The size of the inclusions and the number of cells containing them increased until 2 days after virus inocula-

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EXPLANATION OF PLATE.

FIG. 1.—Normal tissue culture of rabbit corneal endothelium. A mitotic figure is seen in the right upper corner of the picture. ×200. H and E.

FIG. 2.—Degenerative changes of tissue culture of corneal endothelial cells 24 hr. after inoculation of NDV. ×200. H. and E.

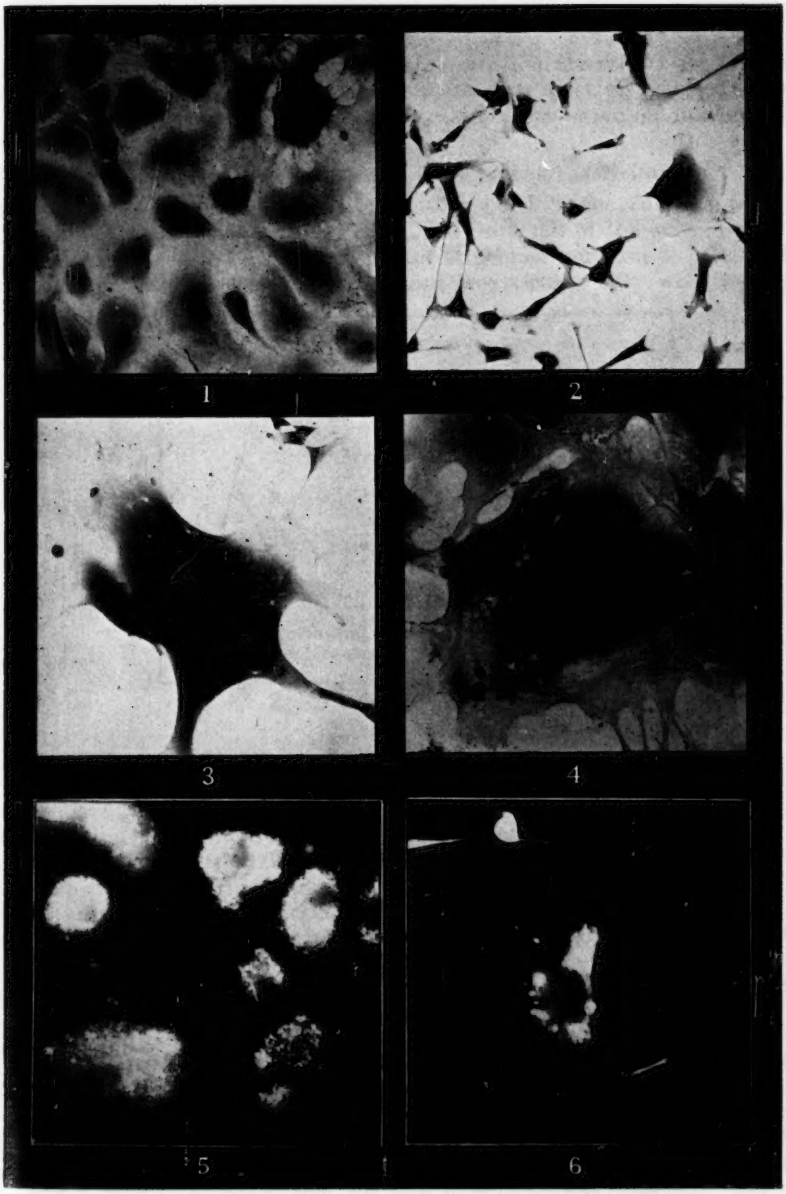
FIG. 3.—Multinucleated cell in tissue culture of corneal endothelium 24 hr. after inoculation of NDV. ×200. H. and E.

FIG. 4.—Intracytoplasmic eosinophilic inclusions (I) in a tissue culture cell of corneal endothelium 30 hr. after inoculation of NDV. ×475. H. and E.

FIGS. 5 and 6.—Tissue cultures of corneal endothelium treated with fluorescein-labelled antiserum (indirect method).

FIG. 5.—Cells 24 hr. after inoculation of NDV. ×300.

FIG. 6.—Cell 48 hr. after inoculation of NDV. Large aggregates of fluorescent granules are seen in cytoplasm. ×475.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tion. Thereafter the number of cells with inclusions decreased rapidly probably due to degenerative changes of the cells.

Generally speaking inclusions were seen frequently in the cells at the periphery of the culture where the degenerative changes were most pronounced. These cells with inclusions were distributed sparsely and were never present in abundance.

Multiplication of NDV in tissue cultures of rabbit corneal endothelium

Two tubes of 10-day-old corneal endothelial cell tissue cultures were inoculated with approximately 10^7 PFU of NDV in 1 ml. of the culture medium and were incubated at 37° for 1 hr. to permit attachment of virus to cells. After incubation, the cells were treated to remove free virus. They were washed 4

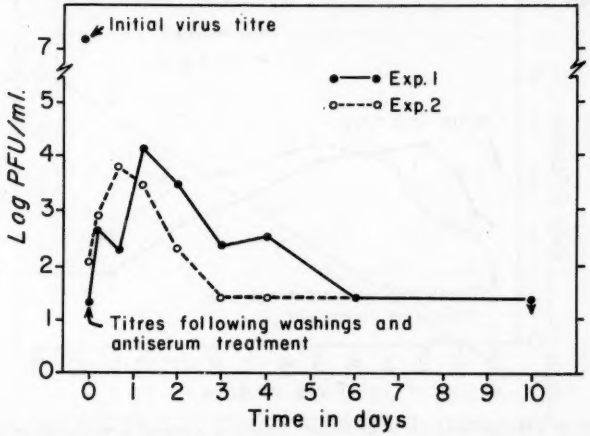

FIG. 7.—Titre of extracellular virus in tissue culture of corneal endothelial cells of rabbits inoculated with $1{\cdot}3 \times 10^7$ PFU of NDV.

times with 1 ml. of medium 199 followed by 1 hr. of incubation at 37° with 1 ml. of a 1/10 dilution of anti-NDV rabbit serum in medium 199. Then the cells were again washed 4 times with medium 199, and incubated at 37° with 1 ml. of fresh culture medium. At various intervals during the incubation period, the cultures were examined for cytopathic effects and 0·1 ml. of the tissue culture medium was removed from each tube for assay of virus infectivity and haemagglutinin. The removed medium was replaced by the same amount of fresh culture medium. The results of this experiment are presented in Fig. 7.

An immediate drop from the initial virus titre of $1{\cdot}3 \times 10^7$ PFU to $2{\cdot}5 \times 10^1$ PFU and $1{\cdot}2 \times 10^2$ PFU was observed at 0 hr. after the washings and antiserum treatment of the tissue cultures. An increase of virus in the culture medium appeared to begin within 5 hr. A peak titre of 8×10^3 PFU was reached after 10 hr. in one experiment and of $1{\cdot}6 \times 10^4$ PFU after 31 hr. in the other. These peak titres were immediately followed by a rather rapid decline in virus titre, and 3 and 6 days after inoculation, no viral infectivity was detected. Haemagglutinin was not found in the medium throughout the experiment. A cytopathic change from polygonal cell type to spindle type, was first observed 19 hr. after

infection and was followed by a gradual destruction of the cells. On the 4th and 6th days, the cytopathic effect of the virus was so extensive that only a few spindle-shaped cells were present on the glass surface and no cells were found thereafter.

When the cultures were inoculated with a lesser amount of virus, such as $2{\cdot}3\times10^3$ PFU, in the same manner as in the preceding experiment, the titres of virus in the medium immediately after the washings and antiserum treatment were about the same as those in the preceding experiment; $2{\cdot}5\times10^1$ PFU and $1{\cdot}2\times10^2$ PFU. Peak titres of $4{\cdot}8\times10^3$ and $5{\cdot}8\times10^3$ PFU were obtained 2 and 5 days respectively after virus inoculation (Fig. 8). These peak titres were

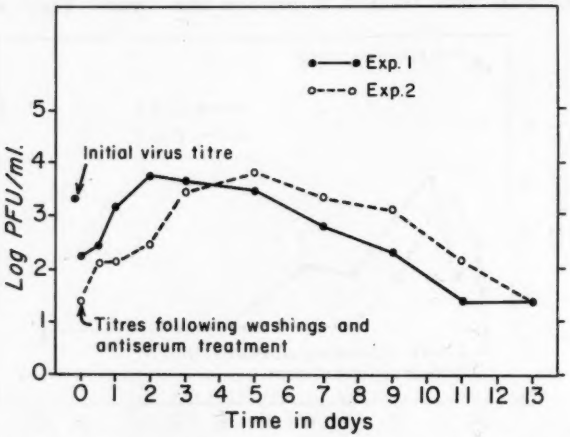

FIG. 8.—Titre of extracellular virus in tissue culture of corneal endothelial cells of rabbits inoculated with $2{\cdot}3\times10^3$ PFU of NDV.

maintained fairly well for 3–4 days with a gradual decrease thereafter. No virus infectivity was found after 13 days. No haemagglutinin was detected throughout the entire period of this experiment. The cytopathic effects began on the 9th day and almost complete destruction of the cells was observed on the 13th day.

With an inoculum of 2×10^2 PFU of NDV, no increase of virus titre was observed for a period of 20 days after infection even though the cells underwent a moderate degree of degeneration.

Thermal inactivation of NDV in medium without corneal endothelial cells

To evaluate the extent of multiplication of NDV in tissue cultures of the corneal endothelial cells of rabbits, the rate of virus inactivation in the culture medium without cells was determined. The virus ($5{\cdot}8\times10^8$ PFU) was added to fresh culture medium, and was incubated at 37° for up to 8 days. During the incubation period, 0·1 ml. of the medium was taken daily from the tube and stored at −35° for virus assay. The thermal inactivation curve is given in Fig. 9.

It appeared that the virus was inactivated at an exponential rate. From this curve the half life of the virus at 37° in this medium was found to be approximately .12 hr. The rate of thermal inactivation of the haemagglutinin was also examined by using the same specimens used in the above experiment. The results showed that the original haemagglutinin titre of 160 in the medium was maintained for 24 hr. Thereafter the titre dropped to 80 and remained unchanged for 6 days.

Development of viral antigens in corneal endothelial cells infected with NDV demonstrated with fluorescein-labelled antiserum

Corneal endothelial cell cultures were inoculated with 10^8 PFU of NDV and incubated at 37° for 30 min. After the incubation period, the inoculum was

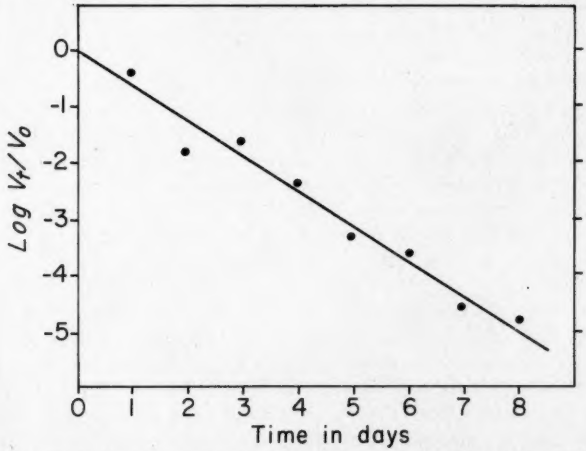

FIG. 9.—The thermal inactivation of NDV at 37° in the culture medium for corneal endothelial cells of rabbits

V_0 = original titre of infectious virus.
V_t = titre of infectious virus remaining at time t.

removed, the cell sheet was washed with medium 199, and 1 ml. of fresh culture medium was added. The cultures were examined with the aid of fluorescein-labelled antiserum at 1, 4, 7, 13, 24 and 48 hr. after inoculation. Viral antigen was first visible in a few cells 7 hr. after inoculation. Infected cells contained small, green-fluorescing granules in their cytoplasm, and in some cells fluorescent granules were concentrated at the periphery of the nuclei. At 13 hr. about 50 per cent of the cells showed fluorescent granules in their cytoplasm, and occasional cells contained fairly large aggregates of fluorescent granules. At 24 and 48 hr., the cytoplasm of almost all cells was filled with dense fluorescent granules of varying sizes (Fig. 5). In several cells the cytoplasm contained one or two large dense aggregates of fluorescing granules, sometimes equal in size to that of the nucleus (Fig. 6). Throughout the period of observation, fluorescent granules were not found in the nuclei of the infected cells.

Three controls consisted of uninfected cells treated with both anti-NDV rabbit serum and fluorescein-labelled anti-rabbit-γ-globulin goat serum, cells infected with NDV treated with both normal rabbit serum and fluorescein-labelled anti-rabbit-γ-globulin goat serum, and cells inoculated with normal allantoic fluid treated with both anti-NDV rabbit serum and fluorescein-labelled anti-rabbit-γ-globulin goat serum. They showed only grey-blue autofluorescence.

DISCUSSION

In tissue cultures of rabbit corneal endothelium infected with NDV, degenerative changes were followed by cell destruction. In addition two characteristic effects were observed; eosinophilic intracytoplasmic inclusions and multinucleated cells. The presence of inclusions in other types of cells inoculated with NDV has been reported (Adams and Prince, 1957; Brandt, 1958). Whether the inclusions induced by NDV contain virus particles or are merely a morphological residuum of the virus infection remains uncertain. In the experiment with the fluorescent antibody technique, some cells contained well-outlined, irregularly shaped, highly-fluorescing bodies in their cytoplasm 24 and 48 hr. after virus was inoculated. This suggests that the inclusions seen in the cells stained with hematoxylin and eosin may represent aggregates of viral particles or antigens. Further studies are required to evaluate this properly.

The formation of multinucleated cells in various tissue culture cells infected with myxoviruses other than NDV has been described and various mechanisms postulated by other observers. Henle, Deinhardt and Girardi (1954), and Russell and Morgan (1959) have attributed the formation of multinucleated cells by mumps virus to the cytolytic property of the virus. Lépine, Chany, Droz and Robbe-Fossat (1959), however, considered the giant cell formation by HA and CA viruses to be the result of a cell agglutination or conglomeration that they described as cytophagia. In the present investigation, tissues cultures of corneal endothelial cells infected with NDV showed what appeared to be fusions of many cells in early stages of infection and aggregation of the nuclei of the fused cells in a later stage.

The formation of multinucleated cells in cultures of corneal endothelium infected with NDV was not observed regularly. The cause of this inconsistency is not understood. Reissig, Black and Melnick (1956) showed that glutamine inhibited the formation of the multinucleated giant cells in cultures of human carcinoma cells infected with either measles or foamy viruses. In the case of the measles and herpes simplex viruses, giant cell formation appears to be related to the strain of virus inoculated (Seligman and Rapp, 1959; Scott and McLeod, 1959).

The evidence obtained from the present experiments supports the conclusion that virus multiplication occurred in rabbit corneal endothelial cells in tissue culture. The possibility that the observed rise in titre was due to elution of virus from the cells rather than to actual multiplication is disproven by the following experimental results: The base line titre of the virus after inoculation remained low over 5 or 12 hr. periods and was followed by a 1·5–2-log increase in titre in all experiments. In the cells inoculated with $2{\cdot}3 \times 10^3$ PFU, the peak titres obtained 2 and 5 days after virus inoculation were almost a half log higher than the titre of the original inoculum and were sustained over a 4-day period.

Recall that the half life of the virus in the culture medium without cells was approximately 12 hr. In view of these results and the fact that cytopathic effects were observed in the cells, and that positive findings were obtained with the fluorescent antibody technique, it is highly probable that infectious virus was actually synthesized in at least some of the cells. Whether NDV multiplies in corneal endothelium of rabbit eye *in vivo* is still to be determined.

By means of the fluorescent antibody technique, viral antigen was demonstrated exclusively in the cytoplasm of the NDV-infected cells. A similar observation was made by others using NDV in various tissue culture systems (Prince and Ginsberg, 1957; Wheelock and Tamm, 1959; Tanaka, Yamaguchi and Kobayashi, 1959; Traver, Northrop and Walker, 1960). These findings are in sharp contrast to those with influenza (Wheelock and Tamm, 1959; Traver *et al.*, 1960) and fowl plague viruses (Breitenfeld and Schäfer, 1957) which show prominent intranuclear antigen production, demonstrable with fluorescent antibody, during their multiplication cycles in tissue cultures. It is possible that NDV antigen produced within the nucleus does not combine with antibody under the condition of the fluorescent antibody technique. The other possibility is that the entire cycle of virus synthesis is carried on exclusively in the cytoplasm of the infected cells.

SUMMARY

The cultivation and cytopathic effects of Newcastle disease virus (NDV) in tissue cultures of rabbit corneal endothelium have been described. The experimental evidence indicated that the tissue cultures were capable of supporting synthesis of new viral antigen and multiplication of infectious virus. The viral antigen demonstrated by fluorescent antibody was observed exclusively in the cytoplasm of the infected cells.

NDV produced degenerative changes in the cells as well as characteristic eosinophilic intracytoplasmic inclusions and multinucleated cells. The latter did not appear regularly in cells infected with the virus.

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STUDIES ON THE PHYSIOLOGICAL DISTURBANCES OCCURRING IN EXPERIMENTAL RABBIT POX: AN APPROACH TO RATIONAL THERAPY

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THE efficiency of chemotherapeutic drugs and antibiotics in the treatment of bacterial infections emphasises the total absence of any effective therapy for those diseases caused by viruses. That vaccines are available for prophylaxis in an increasing number of these diseases is of no comfort to those persons unfortunate enough to become infected. For the many mild and self-limiting diseases this therapeutic impotence is unimportant, but for the patient with such a lethal disease as smallpox it is tragic that treatment is still limited to prayer, sympathy and supportive nursing.

The suspicion that this need not be so was first aroused by some observations made while studying the pathogenesis of rabbit pox as a model for human smallpox. These observations suggested that rabbits dying from this disease do so because of an acute adrenal failure. The experiments reported below, although refuting this hypothesis, have yielded information about the physiological changes occurring in this disease which suggests that the goal of an effective therapeutic attack on virus diseases is not unattainable. The experiments so far have raised more questions than they have answered, but it is felt that the few positive findings that have emerged are of sufficient interest to justify the publication of this report.

MATERIALS AND METHODS

Animal technique

Rabbits, of an average weight of 2·5 kg. and of various breeds and either sex, were obtained from Allington Farm, Porton. They were inoculated via the marginal ear vein with 1·0 ml. of virus suspension, diluted to contain about 30 pfu. (pock-forming units); blood samples were also removed from this vein as required. Throughout the experiments the rabbits had free access to water and pellet food.

Virus

Two strains of rabbit pox virus have been used. The earliest experiments were made with the Rockefeller Institute strain (Pearce, Rosahn and Hu, 1936), obtained from Dr. R. Shope, but the majority of the experiments have been carried out with the Utrecht strain (Jansen, 1942) which was kindly supplied by Professor F. Fenner. Both strains were given a series of intra-testicular passages in rabbits before use, and all experiments with any one strain were carried out with a single batch of virus.

Physiological and chemical procedures

Body temperature.—The rectal temperature was measured with an ordinary clinical thermometer.

Blood pressure.—The indirect method described by Grant and Rothschild (1934) was used.

Blood chemistry.—Urea and sugar were estimated by the methods described by Delory (1949), using the Hilger Biochem absorptiometer. Plasma sodium and potassium were measured by flame photometry on samples of heparinized plasma (0·1 mg. heparin/ml. blood).

Urine analysis.—Specific gravity was determined by the density gradient column method (Lowry and Hunter, 1945) and urea by the hypobromite method (Harrison, 1947). Potassium and sodium were estimated by flame photometry.

Drugs

Metaraminol bitartrate (" Aramine ") was obtained from Merck, Sharpe and Dohme Ltd., mephentermine sulphate (" Mephine ") from John Wyeth and Brother Ltd., l-noradrenaline (" Levophed ") from Bayer Products Ltd., methoxamine hydrochloride (" Vasoxine ") and triprolidine hydrochloride (" Actidil ") from Burroughs Wellcome and Co. and chlorpromazine hydrochloride (" Largactil ") from May and Baker Ltd. Hydrocortisone hemisuccinate (" Ef-Cortelan Soluble ") was supplied by Glaxo Laboratories Ltd., thenalidine tartrate (" Sandosten with Calcium ") by Sandoz Products Ltd. and phenoxybenzamine (" Dibenyline ") by Smith, Kline and French Laboratories Ltd.

RESULTS

Adrenal Function

While studying the pathogenesis of rabbit pox the concurrence of a high concentration of virus in the adrenals (Westwood, Boulter, Bowen and Maber, unpublished) with the presence of focal necrosis in the cortex of that organ (Lancaster, unpublished) was noted. In Table I some of the clinical signs observed in rabbits and monkeys dying from rabbit pox and smallpox respectively are compared with those known to follow adrenalectomy. Most of these are admittedly of a non-specific nature and occur in many acute infections, but the terminal fall in body temperature to sub-normal levels, together with the pathological changes in the adrenal cortex, suggested that they were, in fact, signs of an acute adrenal failure. If this were so, replacement therapy with corticosteroids until adrenal function was restored might be life-saving. As a test of this hypothetical adrenal failure we looked for the blood changes listed in Table I.

TABLE I.—*Signs Present in Rabbits and Monkeys Dying from Rabbit Pox and Smallpox Respectively Compared with Those Produced by Adrenalectomy*

Signs	Adrenalectomy	Rabbit	Monkey
Loss of appetite	+	+	+
Diarrhoea	+	+	—
Loss of weight	+	+	+
Weakness	+	+	+
Hypotension	+	?	.
Fall in body temp.	+	+	+
Fall in plasma Na	+	.	.
Fall in blood sugar	+	.	.
Rise in plasma K	+	.	.
Rise in blood urea	+	.	.

\+ Indicates sign present.
— Not known.
? Hypotension suspected.

The findings in 4 rabbits are illustrated in Fig. 1. Both blood urea and plasma potassium concentrations rose as expected, but there was no corresponding fall in glucose or sodium. On the contrary, there was a tendency for the glucose con-

centration to rise. These findings have been repeatedly confirmed in subsequent experiments and clearly exclude the presence of a simple adrenal failure.

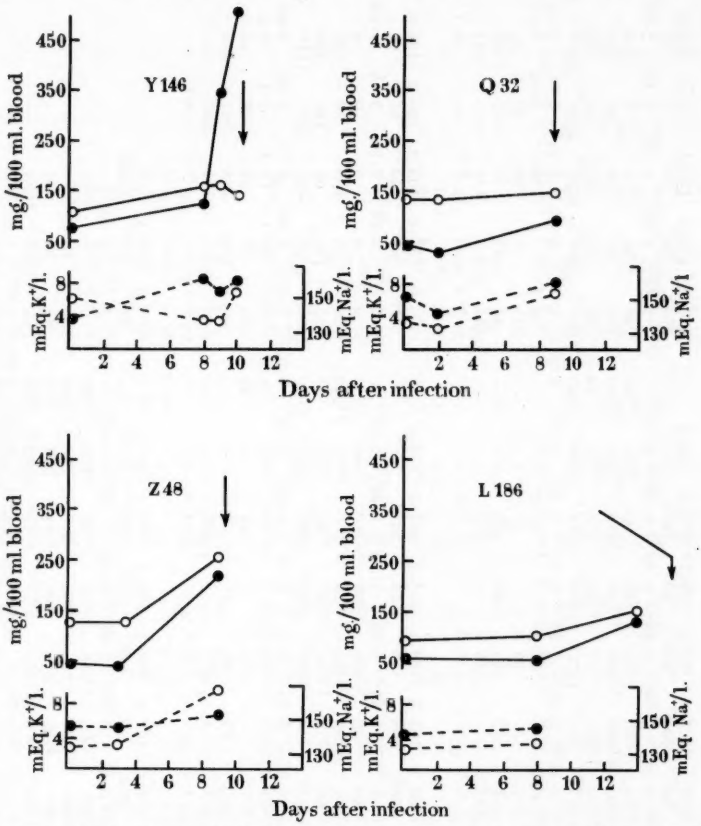

FIG. 1.—Changes in blood urea, blood glucose, plasma sodium and plasma potassium in rabbit pox. The time of death is indicated by the arrows; it occurred spontaneously in Rabbits Y146 and Z48 but followed cardiac puncture in the others.

Blood urea ●——● Plasma Na^+ ●- - - -●
Blood glucose ○——○ Plasma K^+ ○- - - -○

Renal Function

The biochemical picture of nitrogen retention and a tendency for plasma potassium to rise suggested that renal failure might be present. To investigate this aspect detailed observations were made of 6 rabbits kept in metabolism cages. Daily records of food and water intake, urine output, blood pressure, body weight and temperature were kept. Urinary specific gravity and concentrations of urea, potassium and sodium were measured, and the blood levels of the last 3 substances were also determined.

TABLE II.—*Physiological Changes in Rabbit Pox*

	Day of disease												
	0	1	2	3	4	5	6	7	8	9	10	11	12
RABBIT B221:													
Temp. (°F.)	102·4	102·2	102·6	102·7	102·4	103·0	—	105·6	105·4	104·4	104·8	104·2	102·4
B.P. (mm. Hg)	98	100	92	92	86	—	—	94	98	70	76	82	80
Weight (kg.)	2·74	2·70	2·64	2·66	2·68	2·68	—	2·64	2·60	2·48	2·40	2·40	2·34
Food (g.)	80	80	80	120	90	120	60	40	20	0	0	0	20
Water (ml.)	180	160	200	160	170	150	120	120	90	50	80	80	120
Urine volume (ml.)	106	103	117	86	108	71	74	44	74	45	48	59	97
„ S.G.	1027	—	1028	1034	1029	1036	1045	1047	1043	1042	1050	1050	1040
„ urea (g./100 ml.)	3·19	3·12	2·41	2·78	2·40	2·91	3·92	4·37	4·45	5·28	6·60	7·24	5·45
„ Na (m.Eq./l.)	20·0	—	37·0	—	35·7	—	—	40·4	—	38·7	9·13	6·52	2·61
„ K. (mEq./l.)	151	—	90	—	87	—	—	117	—	89	98	85	102
Blood urea (mg./100 ml.)	43	41	35	36	29	—	—	44	47	70	72	92	—
Plasma Na (mEq./l.)	152	—	155	—	172	—	—	147	—	151	—	151	—
„ K. (mEq./l.)	2·9	—	2·6	—	3·4	—	—	3·4	—	3·4	—	4·7	—
RABBIT D207:													
Temp. (°F.)	104·4	102·6	102·4	102·2	103·6	103·8	—	105·6	105·6	105·2	105·2	103·6	103·6
B.P. (mm. Hg.)	90	106	76	86	104	—	—	82	68	80	68	64	72
Weight (kg.)	2·26	2·26	2·30	2·30	2·34	2·32	—	2·26	2·20	2·16	2·10	1·98	2·04
Food (g.)	80	80	100	60	80	80	80	0	0	0	0	0	20
Water (ml.)	170	190	180	130	150	120	140	50	80	100	90	120	150
Urine volume (ml.)	86	72	86	87	86	77	87	53	110	69	136	130	82
„ S.G.	1027	—	1031	1034	1039	1045	1050	1030	1028	1015	1017	1017	1015
„ urea (g./100 ml.)	3·07	3·84	3·01	3·59	4·05	3·84	3·92	3·79	3·46	2·74	2·95	2·99	2·96
„ Na (mEq./l.)	71·7	—	54·8	—	79·2	—	84·8	73·9	39·1	13·1	4·4	2·2	3·0
„ K. (mEq./l.)	107	—	100	—	149	—	143	103	72	43	43	42	37
Blood urea (mg./100 ml.)	41	37	38	41	29	—	—	57	110	118	181	174	—
Plasma Na (mEq./l.)	148	—	150	—	134	—	—	146	—	145	146	—	—
„ K. (mEq./l.)	4·5	—	3·7	—	4·0	—	—	6·7	—	7·4	5·0	—	—
RABBIT B256:													
Temp. (°F.)	102·6	102·4	103·2	103·1	104·0	105·4	—	105·0	103·4	—	—	—	—
B.P. (mm. Hg)	90	86	90	80	76	—	—	72	40	Dead	—	—	—
Weight (kg.)	2·84	2·85	2·85	2·78	2·82	2·80	—	2·74	2·60	—	—	—	—
Food (g.)	100	100	110	50	90	100	40	0	0	—	—	—	—
Water (ml.)	400	350	350	240	270	230	240	130	10	—	—	—	—
Urine volume (ml.)	270	232	242	190	168	218	178	87	53	2	—	—	—
„ S.G.	1015	—	1015	1017	1022	1013	1017	1012	1014	1031	—	—	—
„ urea (g./100 ml.)	1·64	1·70	1·75	1·86	2·11	1·58	1·69	1·97	1·92	1·75	—	—	—
„ Na (mEq./l.)	8·7	—	23·1	—	32·6	—	31·7	12·2	12·2	35·7	—	—	—
„ K. (mEq./l.)	57	—	57	—	76	—	34	44	33	162	—	—	—
Blood urea (mg./100 ml.)	38	34	40	49	35	—	—	70	222	356	—	—	—
Plasma Na (mEq./l.)	145	—	151	—	150	—	—	145	145	—	—	—	—
„ K. (mEq./l.)	3·2	—	4·8	—	3·2	—	—	3·2	5·5	—	—	—	—

Day of disease

	Day of disease												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Rabbit D100													
Temp. (°F.)	101·8	102·2	102·6	102·4	103·2	105·6	—	105·6	103·8	—	—	—	—
B.P. (mm. Hg)	84	80	76	88	96	—	—	68	36	Dead	—	—	—
Weight (kg.)	3·18	3·14	3·12	2·98	2·92	2·84	—	2·74	2·62	—	—	—	—
Food (g.)	100	80	50	20	50	40	0	10	0	—	—	—	—
Water (ml.)	390	250	340	260	230	160	170	120	40	10	—	—	—
Urine volume (ml.)	295	250	150	307	285	156	126	125	45	12	—	—	—
„ S.G.	1015	—	1013	1009	1017	1018	1019	1019	1018	1023	—	—	—
„ urea (g./100 ml.)	1·73	1·75	1·65	1·39	2·30	2·62	2·79	3·34	2·64	1·81	—	—	—
„ Na (mEq./l.)	—	—	23·5	—	28·3	—	33·9	24·4	17·0	23·5	—	—	—
„ K. (mEq./l.)	—	—	50·3	—	47·9	—	58·2	43·3	39·5	56·9	—	—	—
Blood urea (mg./100 ml.)	46	43	43	47	28	—	—	52	174	236	—	—	—
Plasma Na (mEq./l.)	204	—	146	—	183	—	—	145	141	—	—	—	—
„ K. (mEq./l.)	2·4	—	3·4	—	5·6	—	—	4·4	6·9	—	—	—	—
Rabbit D104:													
Temp. (°F.)	102·8	102·6	102·7	103·0	106·0	106·6	—	106·2	102·4	Dead	—	—	—
B.P. (mm. Hg)	74	76	80	80	80	—	—	60	32	—	—	—	—
Weight (kg.)	3·16	3·14	3·20	3·20	3·18	3·12	—	3·04	2·84	—	—	—	—
Food (g.)	120	120	140	100	90	40	0	20	0	—	—	—	—
Water (ml.)	250	290	310	190	180	60	120	130	130	—	—	—	—
Urine volume (ml.)	226	162	162	166	132	67	87	82	104	11	—	—	—
„ S.G.	1016	—	1023	1022	1036	1039	1030	1031	1024	1033	—	—	—
„ urea (g./100 ml.)	2·12	2·57	2·29	2·21	3·17	4·34	3·91	3·60	3·09	3·62	—	—	—
„ Na (mEq./l.)	40·9	—	37·8	—	76·1	96·5	45·6	16·5	14·4	43·5	—	—	—
„ K. (mEq./l.)	79	—	79	—	157	176	82	59	40	47	—	—	—
Blood urea (mg./100 ml.)	40	35	38	37	43	—	—	59	89	118	—	—	—
Plasma Na (mEq./l.)	145	—	150	—	141	—	—	145	131	—	—	—	—
„ K. (mEq./l.)	3·7	—	3·7	—	4·2	—	—	5·3	36·5	—	—	—	—
Rabbit D107:													
Temp. (°F.)	103·4	102·4	102·2	101·8	102·4	105·0	—	106·4	106·4	104·6	104·6	104·4	—
B.P. (mm. Hg)	100	84	92	100	90	—	—	100	80	38	84	104	Dead
Weight (kg.)	3·12	3·14	3·11	3·20	3·18	3·10	—	3·00	2·90	2·76	2·80	2·66	—
Food (g.)	100	100	160	80	110	40	40	0	0	0	0	0	—
Water (ml.)	180	180	250	150	160	100	130	50	50	40	100	40	—
Urine volume (ml.)	90	70	78	77	91	85	54	81	72	63	32	35	2–4
„ S.G.	1032	—	1051	1054	1047	1038	1043	1026	1030	1024	1042	1050	1014
„ urea (g./100 ml.)	4·60	5·50	4·78	5·15	4·47	4·23	5·97	4·47	4·04	4·59	7·48	8·88	2·15
„ Na (mEq./l.)	67·4	64·4	73·0	107·5	108·8	—	—	27·4	—	17·4	13·1	15·2	8·7
„ K. (mEq./l.)	205	240	238	217	219	—	—	105	—	45	81	131	35
Blood urea (mg./100 ml.)	44	46	49	43	43	—	—	56	84	124	112	150	—
Plasma Na (mEq./l.)	155	—	157	—	155	—	—	154	—	165	—	155	—
„ K. (mEq./l.)	42	—	3·4	—	3·2	—	—	5·3	—	4·7	—	5·3	—

The relevant data are given in Table II. Rabbits B 221 and D 207 survived but the others died on the days stated. The intake of both food and water declined from the commencement of pyrexia and there were corresponding falls in body weight and urine output. Since the kidneys compensated for this diminishing urine output by excreting a more concentrated urine, as shown by the rises in urea and specific gravity, it was concluded that kidney function was unimpaired and that the uraemia was not of renal origin.

Two other significant findings emerged from this experiment. Firstly, all animals showed a fall in blood pressure around the 7th to 9th day after infection;

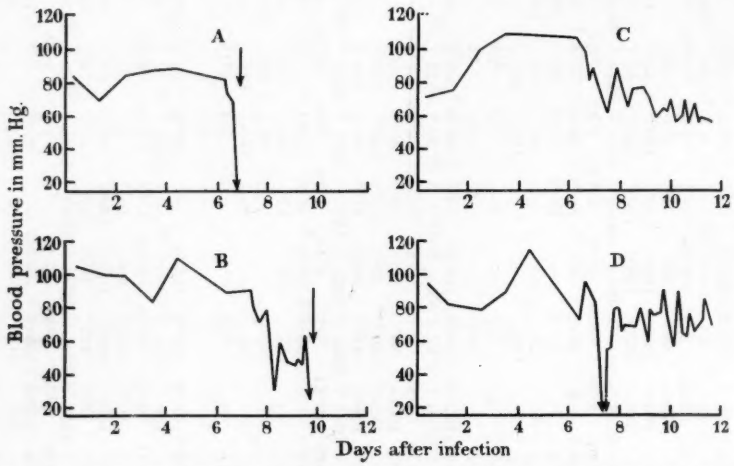

FIG. 2.- Blood pressure changes in rabbit pox: the time of death is indicated by the arrows.

in fatal cases this fall was severe and suggested the occurrence of a shock-like state. Secondly, in the only post-mortem sample available (Rabbit D 104), the plasma potassium had risen to the high level of 36·5 mEq./l.

The Significance of Hypotension

The blood pressure changes in the previous experiment suggested that death or survival might depend on the degree and duration of hypotension. To test this suggestion the blood pressures of 19 rabbits were recorded every few hours throughout the day and night, from the 7th to the 12th post-infection day inclusive. Outside this period of intensive observation only single daily observations were made.

Five rabbits survived, the remainder dying between the 7th and 10th day after infection. All animals, both survivors and fatal cases, had a fall in blood pressure at a time which varied from 48 hr. to as late as 5 days after the onset of pyrexia. It was found that the blood pressure changes fell into 4 distinct groups, illustrated in Fig. 2. Fatal cases invariably had a profound fall in pressure, often to unrecordable levels. In some the fall was precipitate and death followed in a

few hours (Fig. 2A). In others the fall was more gradual, with a subsequent partial restoration of pressure to about 60 mm./Hg until death supervened a day or so later (Fig. 2B). These two groups were further distinguished by the incoagulability of the blood at death of the rabbits in the first group, whereas the blood of the animals in the second group clotted normally. Surviving rabbits did not have such profound and prolonged hypotension. In 4 animals the pressure fluctuated around 50–60 mm./Hg, the lowest levels recorded for each rabbit being 57, 54, 40 and 38 mm./Hg respectively, and this only for a brief period (Fig. 3C). In the other rabbit there was a short time in which the pressure became unreadable, but it rapidly rose again to the 70–80 mm./Hg range (Fig. 3D).

The Significance of Raised Plasma Potassium

Fig. 1 shows that there is a regular, but moderate, rise in plasma potassium during the course of the disease. In the only post-mortem sample available (Rabbit D 104, Table II) the concentration of potassium was 36·5 mEq./l. This level is almost certainly lethal and indicated that death might be due to potassium poisoning. However, although the animal was still warm and flaccid when the blood was removed, ignorance of the actual time of death prevented post-morten leakage of potassium from cells being excluded in this instance.

The constant day and night watch necessitated by the previous experiment enabled blood to be collected at death, which was signalled by the noise of the agonal convulsions. Eleven samples of heparinized blood were obtained; these were centrifuged immediately and the plasma stored at −20° until tested. The individual results, given in Table III, ranged from 8·3 to 31·4 mEq./l. In order

TABLE III.—*Plasma Potassium in Fatal Rabbit Pox Infection*

Rabbit	Day of death	K^+ in mEq./l.
H113	7	14·7
H126	7	30·5
233C	8	10·2
H134	8	10·8
H85	9	31·4
227H	9	9·7
282G	9	15·1
H37	9	11·0
H146	9	12·4
H66	9	8·3
286G	10	13·4

to assess the significance of these figures as indicative of death from potassium poisoning it is necessary to know the lethal range of plasma potassium levels in rabbits. Tabor and Rosenthal (1945) injected normal rabbits with potassium chloride at a slow and constant rate intravenously and sampled the heart blood at the moment of cardiac arrest. The levels of plasma potassium that they found are compared with our findings in rabbit pox in Table IV. The similarity is striking and it is difficult not to conclude that our rabbits died from potassium intoxication. It is of interest to note that these authors found that shock, induced by haemorrhage in this instance, increased susceptibility to potassium. Similar findings have been reported by Winkler, Hoff and Smith (1938); the other results included in Table IV indicate that the toxic level of the ion is of the same order in the

TABLE IV.—*Plasma Potassium in Fatal Rabbit Pox and Potassium Poisoning*

Animal	Treatment	Number of animals	K^+ in mEq./l Mean	K^+ in mEq./l Range	References and remarks
Rabbit	Normal	13	3·3	2·4–4·4	—
,,	Rabbit pox	11	15·2	8·3–31·4	—
,,	KCl i.v.	7	18·5	10·4–27·0	Tabor and Rosenthal (1945)
,,	Haem + KCl i.v.	9	13·4	12·0–15·3	,, ,, ,,
,,	KCl i.v.	—	—	14–16	Winkler *et al.* (1938)
,,	Isol. Heart + KCl	—	12·0	—	Fibrillation } McLean, Bay and
,,	Heart	—	18·0	—	Cardiac arrest } Hastings (1933)
Dog	KCl i.v.	—	19·7	—	Spurr, Barlow and Lambert (1959)

isolated heart and also in other species. The normal values of plasma potassium found by us in 13 rabbits are also given.

Effects of Empirical Treatment

The preceding experiments indicated that the major events in fatal rabbit pox were the development of a state of shock and the subsequent rise of plasma potassium to lethal levels. Before trying to identify the factors involved in this haemodynamic collapse, we decided to see whether measures designed to modify it had any effect on the outcome of the disease. If successful, this approach would not only attain our objective of a rational therapy for a virus disease, but also might indicate the likely mechanisms involved.

Treatment after occurrence of hypotension

For this purpose 3 long acting pressor amines (metaraminol bitartrate, mephentermine sulphate and methoxamine hydrochloride) and a corticosteroid (hydrocortisone hemisuccinate) were used. The difficulties of maintaining a constant intravenous infusion precluded any large scale use of nor-adrenaline but a single dose of this drug, given to an animal which failed to respond to one of the other pressor amines, was without effect. Frequent observations of temperature and blood pressure were made throughout the day and night, and treatment was begun as soon as the blood pressure began to fall. Injections were repeated at short intervals in the absence of a response. The results, together with details of doses, are given in Table V.

TABLE V.—*Effect of Drugs Given During Hypotension in Rabbit Pox*

Drug	Dose	Mortality
Mephentermine	2·0 mg.	4/4
Methoxamine	0·7 mg.	5/5
Metaraminol	3·6 mg.	2/2
Hydrocortisone	4·0 mg.	2/3
Controls	—	4/4

The pressor amines were completely ineffective in combating the hypotension; in not one instance did the blood pressure rise after the administration of the drug. This is reflected in the 100 per cent mortality in these groups. The response to hydrocortisone was more equivocal. There was one survivor out of the 3 rabbits

receiving this drug alone, a result which can hardly be considered as significant, particularly as none of the animals responded by a rise in blood pressure. However, one particular rabbit (not shown in Table V) did respond dramatically to a very large does of the drug. The animal had been severely hypotensive for several hours despite repeated injections of methoxamine. As it was the last survivor of its group and was apparently following the same fatal course as its companions, we decided to try the effect of a large dose of hydrocortisone. Within a few seconds of an intravenous injection of 20 mg. of the steroid the ear flushed and the blood pressure rose ; this rise continued over the next few hours and the animal survived.

Treatment before occurrence of hypotension

The preceding experiment showed the difficulty in preventing a fatal outcome once the blood pressure had begun to fall. Since this fall might be easier to prevent than to reverse, the following drugs were tested for their value in this respect ; triprolidine hydrochloride (an anti-histamine), dibenyline hydrochloride (an anti-serotonin agent with adrenergic-blocking properties), thenalidine tartrate with added calcium (having both anti-histamine and anti-serotonin properties) and hydrocortisone hemisuccinate. In view of the result in the last experiment this steroid was used at 2 dose levels. Chlorpromazine was also included because of its known protective action against shock induced by such varied mechanisms as endotoxins (Reilly, Compagnon, Tournier and DuBuit, 1954 ; Abernathy, Halberg and Spink, 1957), irradiation (Haley, McCormick and McCullough, 1955), and haemorrhage or " drumming " (Kelan and Zweifach, 1954). The drugs were tested both singly and in various combinations. As the critical factor was likely to be the blood concentrations of the drugs at the moment that the mechanism leading to hypotension was triggered off, and since this could not be predicted, treatment was begun arbitrarily 48 hr. after the onset of pyrexia. Drugs were given, wherever possible, subcutaneously rather than intravenously so as to maintain reasonably constant blood levels. The details of treatment, together with results, are listed in Table VI.

TABLE VI.—*Effect of Drugs Given Before Hypotension in Rabbit Pox*

Drug	Dose	Mortality
Triprolidine	25 mg./8 hr.	4/4
Dibenyline	25 mg./day	4/4
Hydrocortisone	5 mg./8 hr.	5/5
,,	20 mg./8 hr.	3/5
Triprolidine and Dibenyline	25 mg./8 hr. 25 mg./day	5/5
Triprolidine and Dibenyline and Hydrocortisone	25 mg./8 hr. 25 mg./day 5 mg./8 hr.	5/5
Thenalidine	1·5 mg./8 hr.	4/5
Chlorpromazine	5 mg./8 hr.	5/5
Controls	—	8/8

There was 100 per cent mortality in all groups except those receiving the larger dose of hydrocortisone or thenalidine. Dibenyline actually seemed to precipitate death when given to hypotensive rabbits, since 6 of 14 rabbits receiving this drug died in convulsions within a few moments of an injection, whereas only 1 of 28

rabbits receiving other drugs did so. This may be related to the adrenergic blocking properties of Dibenyline.

Thus, of 65 rabbits used in these 2 experiments, there were but 5 survivors, yet 4 of these had received hydrocortisone. Although the probability of this occurring by chance is less than 3 per cent, the value of steroid therapy remained doubtful. Two further experiments, summarised in Table VII, hardly clarified

TABLE VII.—*Effect of Various Dosages of Hydrocortisone on Mortality in Rabbit Pox*

Experiment	Dose/mg.	Mortality
022660	75	2/3
	Controls	3/3
031160	5	3/5
	20	5/5
	40	5/5
	Controls	5/5

the situation; the restriction of survivors in the second experiment to the lowest dose group is particularly puzzling. In both experiments treatment was begun 48 hr. after the rise in temperature and continued every 8 hr. Combining the results of all experiments in which hydrocortisone was given shows that 7 of 37 rabbits given the steroid survived, compared with only 1 of 54 other rabbits. Despite this apparent tenfold increase in the survival rate (from 1·85 per cent to 18·9 per cent) with steroid therapy, the latter is hardly an ideal treatment.

DISCUSSION

These results indicate the following sequence of events after infection with rabbit pox virus. An incubation period of 3 to 6 days is terminated by the onset of pyrexia. From this time a reduced intake of food and water results in loss of weight and a diminishing output of urine. A little later, at a time varying from 48 hr. to as long as 5 days after the rise in temperature, a fall in blood pressure occurs. If the hypotension is either slight or of short duration the animal will survive ; if not, death will follow. Meanwhile the concentrations of blood urea and plasma potassium rise, the latter ion reaching lethal levels in fatal cases. Death follows the hypotension by a period which may be as short as a few hours or as along as several days : it is heralded by a series of convulsive movements of the dying animal. These acute and delayed deaths differ in that the blood is incoagulable in the former but clots normally in the latter group. In survivors the intake of food and water increases, the output of urine rises, the levels of blood urea and potassium fall and the animal regains weight and strength.

The mechanism of the hypotension is unknown. It occurs late in the disease, there is no obvious oedema and nothing to suggest gross vasodilatation or local pooling of blood. It is tempting to attribute it to a viral toxin, for the syndrome has many resemblances to that produced by rickettsial toxin (Cox, 1953 ; Visco, 1957). That pox viruses have a toxic effect has been demonstrated both *in vitro* (Brown, Mayyasi and Officer, 1959) and *in vivo* (Cassel, 1957); the latter example related to a single variant strain of vaccinia. However, although Cassel's strain was toxic at a dose $2{\cdot}3 \times 10^6$ pfu., and the toxic dose of rickettsiae is of the same order, we have been unable to demonstrate any toxicity with our virus using

doses as high as 10^7 pfu. Recent experiments (Appleyard, personal communication) have shown that doses in excess of 10^9 pfu. are still devoid of toxicity. It is more probable that the endogenous mechanisms activated in general inflammation (reviewed by Spector, 1958) are implicated.

The attempts to modify the disease with drugs were not very helpful in indicating possible therapeutic measures, although the results do suggest that neither histamine nor serotonin are likely to be implicated in the hypotension. The only substance of any value was hydrocortisone, and that to a limited extent. Nevertheless, Herrlich (1958) reported that human smallpox, except in the severe purpuric form, responded to cortisone. Steroid therapy in virus diseases is regarded with suspicion and one only has to recall the numerous reports of the enhancement of viral growth and virulence in experimental infections (Kass and Finland, 1953) and the report by Haggerty and Eley (1956), of 12 fatal cases of the usually mild varicella to realise how justified is that suspicion. However there may be a place for the corticosteroids in the treatment of virus infections, that place being in the treatment of shock, if and when it supervenes.

Because of technical difficulties, no attempt was made to reduce the high terminal levels of potassium. If it can be established that similar changes occur in human smallpox, it might be worthwhile using the usual methods of combating this, namely haemo- or peritoneal dialysis, exchange resins, insulin and glucose, etc.

Experiments, to be reported elsewhere, have shown that passively administered antibody will prevent death from rabbit pox even when given as late as 2 days after the onset of pyrexia, but not when given later. This, considered with the findings reported above, suggests that treatment may have to be related to 3 distinct stages of the disease. Substances which interfere with viral multiplication, whether antibody, drug or interferon, are most likely to be effective before the critical stage of hypotension is reached. Once the blood pressure has begun to fall such substances, by themselves, are likely to be useless ; treatment should then be aimed at restoring the blood pressure before the irreversible stage of shock occurs. Before this is feasible more must be learnt of the mechanisms involved, and experiments are being continued with this end in view. If hypotension persists, irreversible shock will ensue ; treatment of this last stage must await the results of the widespread research being carried out on this phenomenon.

SUMMARY

A study has been made of the physiological changes occurring in rabbit pox. These include hypotension, diminished output of urine and increases in the concentrations of blood urea and plasma potassium. No evidence has been found of any functional failure of either adrenal or kidney, despite the large amounts of virus and areas of cortical necrosis in the former organ. The degree and duration of hypotension appears to decide the fate of the animal. If slight, the defence mechanisms eliminate the virus and recovery follows. If not, shock supervenes and the blood urea and potassium rise, the latter ion eventually reaching a lethal concentration. Attempts to modify the disease with various drugs were unsuccessful, except that hydrocortisone had a slight, but definite, effect upon mortality. The implications of these findings with regard to possible therapy in human smallpox are discussed.

Our thanks are due to Sir Charles Lovatt Evans, Professor Maizells and particularly to Dr. A. M. Joekes for their helpful advice to virologists who have strayed into physiological fields. For the estimations of plasma and urinary electrolytes we are indebted to Dr. K. Brown-Grant and Mr. L. C. Thomas of the Chemical Defence Experimental Establishment, Porton. Supplies of metaraminol, mephentermine and triprolidine were generously given by Merck, Sharpe and Dohme Ltd., John Wyeth and Brother Ltd., and Burroughs Wellcome and Co. respectively. The statistical treatment of results was kindly undertaken by our colleague, Mr. S. Peto. Finally, we gratefully acknowledge the encouragement and helpful criticism of Dr. J. C. N. Westwood.

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LOCAL SKIN REACTIVITY TO INFLUENZA VIRUS

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In previous papers from this laboratory some aspects of the toxic agent associated with influenza virus particles have been described (Kato and Hara, 1961; Kato and Okada, 1961). Intravenous injection of the toxic agent into experimental animals causes toxic death with severe generalized damage to the blood capillaries of the internal organs. Recently it has been found that influenza virus particles possess both skin-preparatory and provocative potencies for the local Shwartzman phenomenon. Further studies have, therefore, been made of the principle, associated with influenza virus particles, which is active in the Shwartzman phenomenon.

MATERIALS AND METHODS

Preparation of virus suspensions

The PR8 (A), Lee (B) and Adachi (A2) strains of influenza virus were used. The preparation of the virus suspensions by high-speed centrifugation was as already described (Kato and Hara, 1961). In certain cases the virus suspensions were further purified, either by a cycle of adsorption onto and elution from chicken red cells, or by an aluminum phosphate column method as described previously (Kato and Okada, 1961).

Haemagglutinin (HA) and infectivity titrations

These were identical with those already described (Kato and Hara, 1961). HA titrations were carried out with chicken red cells, and HA titres were expressed as values per ml. Infectivity titres were calculated in terms of EID_{50} per ml.

Treatment of virus suspensions with sonic vibration and repeated freezing and thawing

Sonic vibration was used as already described (Kato and Hara, 1961) for 1 hr. unless otherwise stated.

Repeated freezing and thawing were carried out as follows. The virus suspensions were frozen promptly in a dry ice-ethanol mixture and thawed in a water bath at 37° These treatments were repeated 10 times unless otherwise stated.

Separation of the HA and S fractions

The treated virus suspensions were fractionated into the HA and S fractions by the red cell procedures as described previously (Kato and Hara, 1961).

Preparations of inactive virus

Inactivated virus suspensions were prepared by heat, ultraviolet irradiation and by sodium ethylmercurithiosalicylate (Merzonin, Takeda Pharmaceutical Industries Ltd.) as described previously (Kato and Hara, 1961; Kato and Okada, 1961).

Elicitation of the Shwartzman phenomenon

Rabbits weighing 2·0–2·5 kg. were used. The skin of the abdomen or back of rabbits was widely depilated with barium sulphate. The animals were injected not less than 24 hr.

after depilation when no signs of irritation were seen. The preparatory injections were made intradermally in a dose of 0·2 ml. into each area. The provocative injections were made intravenously 18–20 hr. after the preparatory injections. The skin of rabbits receiving the injections was examined every hr. afterwards. Since the positive reactions became pronounced usually in about 4 hr. after the intravenous injection, the final examination was done 5 hr. after the injection. The degrees of the skin reactions were defined as follows.

– : No skin reaction, or only slight erythema and oedema of the same degree as found just before the provocative injection.
1+ : Marked erythema with oedema.
2+ : Haemorrhage less than 9 mm. diameter.
3+ : Haemorrhage more than 10 mm. diameter, or haemorrhage accompanied by necrosis.

Culture filtrate of Esch. coli

As a control of known activity in the Shwartzman phenomenon culture filtrate of *Esch. coli* was employed. A stock strain, *Esch. coli* BT_2, was inoculated into meat infusion broth, incubated for 96 hr. at 37°, and Seitz filtered. The minimal lethal dose of the filtrate for rabbits weighing 2·0–2·5 kg. was approximately 1·0 ml. per kg. of body weight. The filtrate was stored at −30° before being used.

Histological examinations

Histological examinations of the prepared skin sites of some rabbits were made just before and 5 hr. after the provocative injection. The skin was excised and fixed in 10 per cent formalin. Sections were stained by haematoxylin and eosin.

RESULTS

The skin-preparatory potency of influenza virus

Upper and lower right and left areas of the skin of the abdomen or back of rabbits received the preparatory injections of the PR8 virus suspension, culture filtrate of *Esch. coli* and other, control, materials. Controls used were normal allantoic fluid, 10 per cent emulsions of normal embryos and chorioallantoic membranes from 12-day-old chick embryos, and supernate of infected allantoic fluid after removing the virus particles by high-speed centrifugation (44,000 *g*, 30 min.) and adsorption onto chicken red cells. Eighteen to 20 hr. after the intradermal injections the areas injected with the virus suspensions showed slight erythema and oedema. The areas injected with the culture filtrate of *Esch. coli* showed a little stronger erythema and oedema, and the areas injected with the various control materials showed no skin reaction. As the provocative injection the culture filtrate of *Esch. coli* was given intravenously into each rabbit in a quantity of 0·8 ml. per kg. of body weight. The skin reactions after the intravenous injection are shown in Table I. It is seen that the areas prepared with the PR8 virus suspension having an HA titre higher than 10,240 and with the culture filtrate of *Esch. coli* showed haemorrhage in 5 hr. after the provocative injection. These areas appeared dark blue, glossy and swollen. The haemorrhagic reactions in the areas prepared with the PR8 virus suspension were weak in comparison with those in the areas prepared with the culture filtrate of *Esch. coli*. None of the control materials showed skin-preparatory potency.

The haemorrhagic reaction reached its maximum size in about 4 hr. after the provocative injection. The healing of the haemorrhagic reaction in the areas prepared with the culture filtrate of *Esch. coli* took about a week. On the other hand, the haemorrhagic reaction in the areas prepared with the PR8 virus suspension resolved within 4 days.

TABLE I.—*Reactions at Sites Prepared with Influenza Virus Suspensions and Control Materials 5 Hr. after the Provocative Injection of Culture Filtrate of* Esch. coli

Rabbit number	Preparatory injection: Material	HA/ml.	EID_{50}/ml. (log)	Reaction after provocative injection*: Degree	Diameter of haemorrhage (mm.)
1	PR8	5,120	9·9	−	−
	PR8	10,240	10·2	1+	−
	Coli filtrate	..	..	2+	8×10
	Saline	..	..	−	−
2	PR8	10,240	10·2	2+	4×4
	PR8	20,480	10·5	2+	6×6
	Coli filtrate	..	..	3+	28×28
	Normal allantoic fluid	..	..	−	−
3	Coli filtrate	..	..	3+	25×26
	Chorioallantoic emulsion	..	..	−	−
	Chick embryo emulsion	..	..	−	−
	Supernatant of infected fluid	<2	..	−	−

* As the provocative injection culture filtrate of *Esch. coli* was given intravenously in a quantity of 0·8 ml. per kg. of body weight.

The effect of sonic vibration and repeated freezing and thawing on the skin-preparatory potency of influenza virus

Since the skin-preparatory potency of intact virus particles was rather weak, treatments capable of uncovering this potency were sought. In a previous paper it was reported that sonic vibration for 1 hr. disintegrated influenza virus particles nearly completely into subunits without destroying the toxic property (Kato and Okada, 1961). It has also been found that repeated freezing and thawing uncover the haemolytic activity of Newcastle disease virus (Granoff and Henle, 1954) and of haemagglutinating virus of Japan (*Myxovirus parainfluenzae* 1, Sendai virus) (Yoshida, 1958; Hosaka, 1958). Therefore the effect of both these treatments was examined.

First, experiments were carried out to find the optimal conditions for both treatments. The results of these experiments are shown in Table II. It is seen that both sonic vibration and repeated freezing and thawing uncovered the skin-preparatory potency of PR8 virus, and that the maximum potency was obtained by sonic vibration for 1 hr. or 7–10 cycles of freezing and thawing. So sonic vibration for 1 hr. and 10 cycles of freezing and thawing were employed in subsequent experiments.

Table III shows the results of more detailed experiments. The size of erythema just before the provocative injection in the areas injected with sonically vibrated virus or frozen and thawed virus was larger than that in the areas injected with intact virus. However, the intradermal injection alone could not cause obvious petechial reactions. Skin reactions after the provocative injection of culture filtrate of *Esch. coli* are shown in the Table. It is seen in every experiment that both 1 hr.'s sonic vibration and 10 cycles of freezing and thawing increased the skin-preparatory potency of the PR8 virus suspensions to a considerable degree. Skin-preparatory potency was enhanced to a similar extent by both treatments.

TABLE II.—*Effect of Sonic Vibration and Repeated Freezing and Thawing on the Preparatory Potency of Influenza Virus* (1)

Rabbit number	Preparatory injection: Material	Preparatory injection: HA/ml.	Reaction after provocative injection*: Degree	Reaction after provocative injection*: Diameters of haemorrhage (mm.)
4	PR8	5,120	2+	2×3
	PR8 sonically vibrated for 1 min.	5,120	2+	6×8
	PR8 sonically vibrated for 5 min.	5,120	2+	7×11
	PR8 sonically vibrated for 10 min.	5,120	3+	9×11
	PR8 sonically vibrated for 30 min.	7,680	3+	14×15
	PR8 sonically vibrated for 60 min.	10,240	3+	16×16
	Coli filtrate	..	3+	22×25
5	PR8	20,480	2+	2×2
	PR8 frozen and thawed 1 cycle	20,480	2+	2×2
	PR8 frozen and thawed 3 cycles	20,480	2+	7×7
	PR8 frozen and thawed 5 cycles	20,480	2+	8×8
	PR8 frozen and thawed 7 cycles	20,480	3+	10×10
	PR8 frozen and thawed 10 cycles	20,480	3+	10×10
	Coli filtrate	..	3+	27×30

* The provocative injection was the same as in Table I.

TABLE III.—*Effect of Sonic Vibration and Repeated Freezing and Thawing on the Preparatory Potency of Influenza Virus* (2)

Rabbit number	Preparatory injection: Material	Preparatory injection: HA/ml.	Preparatory injection: EID_{50}/ml. (log)	Reaction after provocative injection*: Degree	Reaction after provocative injection*: Diameters of haemorrhage (mm.)
8	PR8	5,120	9·9	—	—
	Sonically vibrated PR8	10,240	5·9	2+	7×7
	Frozen and thawed PR8	5,120	9·8	2+	4×4
	Coli filtrate	..	..	3+	17×18
9	PR8	10,240	10·2	—	—
	Sonically vibrated PR8	20,480	6·1	3+	15×20
	Frozen and thawed PR8	10,240	10·2	3+	12×15
	Coli filtrate	..	..	3+	18×28
10	PR8	20,480	10·5	2+	2×2
	Sonically vibrated PR8	40,980	6·0	3+	14×14
	Frozen and thawed PR8	20,480	10·5	3+	12×12
	Coli filtrate	..	..	3+	23×24
11	PR8	40,960	10·8	2+	4×5
	Sonically vibrated PR8	40,960	6·7	3+	20×20
	Frozen and thawed PR8	40,960	10·7	3+	25×25
	Coli filtrate	..	..	3+	26×26

* The provocative injection was the same as in Table I.
† Sonic vibration for 1 hr.
‡ Freezing and thawing were repeated 10 times.

The gross appearance of the haemorrhagic reactions (Rabbit 9), shown in Fig. 1, was the same in the areas prepared with the virus preparations and with the culture filtrate of *Esch. coli*, although size of haemorrhagic areas differed. The areas prepared with treated virus suspensions with a pre-treatment titre of 40,960

HA units per ml. showed haemorrhage of similar size to that in the areas prepared with the culture filtrate of *Esch. coli*. The process of healing took a week in both areas.

Most of the infectivity of the virus suspensions was destroyed by sonic vibration for 1 hr.. whereas 10 cycles of freezing and thawing did not significantly change it (Table III). Electron microscopy of the sonically vibrated virus suspensions revealed that the virus particle was disintegrated nearly completely into its subunits as reported previously (Kato and Okada, 1961), whereas 10 cycles of freezing and thawing had not such a disintegrating effect. The following experiments were carried out using both kinds of treated virus suspension to investigate the relation of the skin-preparatory factor to the HA particles.

Purified PR8 virus suspensions treated with 1 hr.'s sonic vibration or 10 cycles of freezing and thawing were separated into HA and S fractions by the red cell procedure. The treated virus suspensions, HA and S fractions were injected intradermally as preparatory materials into rabbits. As the provocative injection culture filtrate of *Esch. coli* was given intravenously in a quantity of 0·8 ml./kg. body weight. The typical result is shown in Table IV. It is seen that

TABLE IV.—*The Relation of the Skin-preparatory Factor to the Haemagglutinin Particles*

	Preparatory injection		Reaction after provocative injection*	
Rabbit number	Material	HA/ml.	Degree	Diameter of haemorrhage (mm.)
12	Sonically vibrated† PR8–RC‡	10,240	3+	12×12
	HA fraction	10,240	2+	4×4
	S fraction	<4	2+	2×2
	Frozen and thawed§ PR8–RC	10,240	2+	8×8
	HA fraction	10,240	2+	7×8
	S fraction	<4	−	−

* The provocative injection was the same as in Table I.
† Sonic vibration for 1 hr.
‡ PR8–RC was PR8 virus suspension purified by the red cell method.
§ Freezing and thawing were repeated 10 times.

following sonic vibration some of the skin-preparatory factor was released from the HA particles and the preparatory potency of the HA particles decreased. On the other hand, such release did not occur following repeated freezing and thawing. These findings are compatible with the results of electron microscopy.

The fate of influenza virus injected into rabbit skin

At various time intervals after the intradermal injections of 0·2 ml. of PR8 virus suspension a piece from one of the injected skin sites about 20 × 20 mm. (wet weight, 2·0 g.) was excised, minced into small pieces, and emulsified to form a 10 per cent suspension. HA and infectivity titrations of the emulsions were made. The results are shown in Table V. It is seen that a rapid drop in virus titre occurred within 6 hr. of the intradermal injection and less than 10^{-4} of the injected dose could be detected 24 hr. after the injection. This rapid disappearance of virus from the injected skin site was probably due to the escape of virus into the lymph and blood circulations. No appreciable amount of non-specific virus inhibitor was found in emulsions of the skin.

TABLE V.—*The Fate of Influenza Virus Injected Intradermally into Rabbits*

Rabbit number	Dose of virus injected into a site*	Interval after injection (hr.)	Virus titres of 10 per cent skin emulsion: HA/ml.	Virus titres of 10 per cent skin emulsion: EID_{50}/ml. (log)	Recovery calculated from EID_{50} (per cent)
14	10,240 HA/ml., $10^{10.4}EID_{50}$/ml. 0·2 ml.	0	32	7·9	31
		3	4	7·0	4
		6	<2	4·5	0·01
		24	<2	3·9	0·003
15	5,120 HA/ml., $10^{10.1}EID_{50}$/ml. 0·2 ml.	0	16	7·5	25
		2	<2	6·9	6
		6	<2	4·7	0·03
		24	<2	4·1	0·01

* The PR8 strain of influenza virus.

The provocative potency of influenza virus

Experiments were made to determine whether influenza virus could also act provocatively. As the preparatory materials PR8, Lee and Adachi suspensions treated with 1 hr. sonic vibration or 10 cycles of freezing and thawing, and culture filtrate of *Esch. coli* were used. As the provocative material PR8 virus suspension was injected intravenously. An approximate minimal lethal dose by the intravenous route of fresh PR8 virus suspension prepared by high-speed centrifugation was 10,240 × 2·5 HA units ($10^{9.8}$ × 2·5 EID_{50}) per kg. body weight (Kato and Hara, 1961). So the provocative injection was made with a dose of 5,120 × 2·5 HA units per kg. body weight. The reactions in the prepared skin sites after the provocative injection are shown in Table VI. It can be seen that the PR8 virus suspension possessed provocative potency. Haemorrhagic reactions could be produced also in sites receiving preparatory injections of immunologically unrelated virus.

EXPLANATION OF PLATES

FIG. 1.—Appearance of haemorrhagic reaction at prepared skin sites, 5 hr. after the intravenous injection of culture filtrate of *Esch. coli* (Rabbit 9). Upper left: Prepared with intact PR8 virus suspension. Upper right: Prepared with sonically vibrated PR8 virus suspension. Lower right: Prepared with frozen and thawed PR8 virus suspension. Lower left: Prepared with the culture filtrate of *Esch. coli*.

FIG. 2.—Histological appearance at skin site injected 20 hr. previously with sonically vibrated PR8 virus suspension. ×48.

FIG. 3.—Higher magnification of the same section as shown in Fig. 2. The upper layer of the skin. ×512.

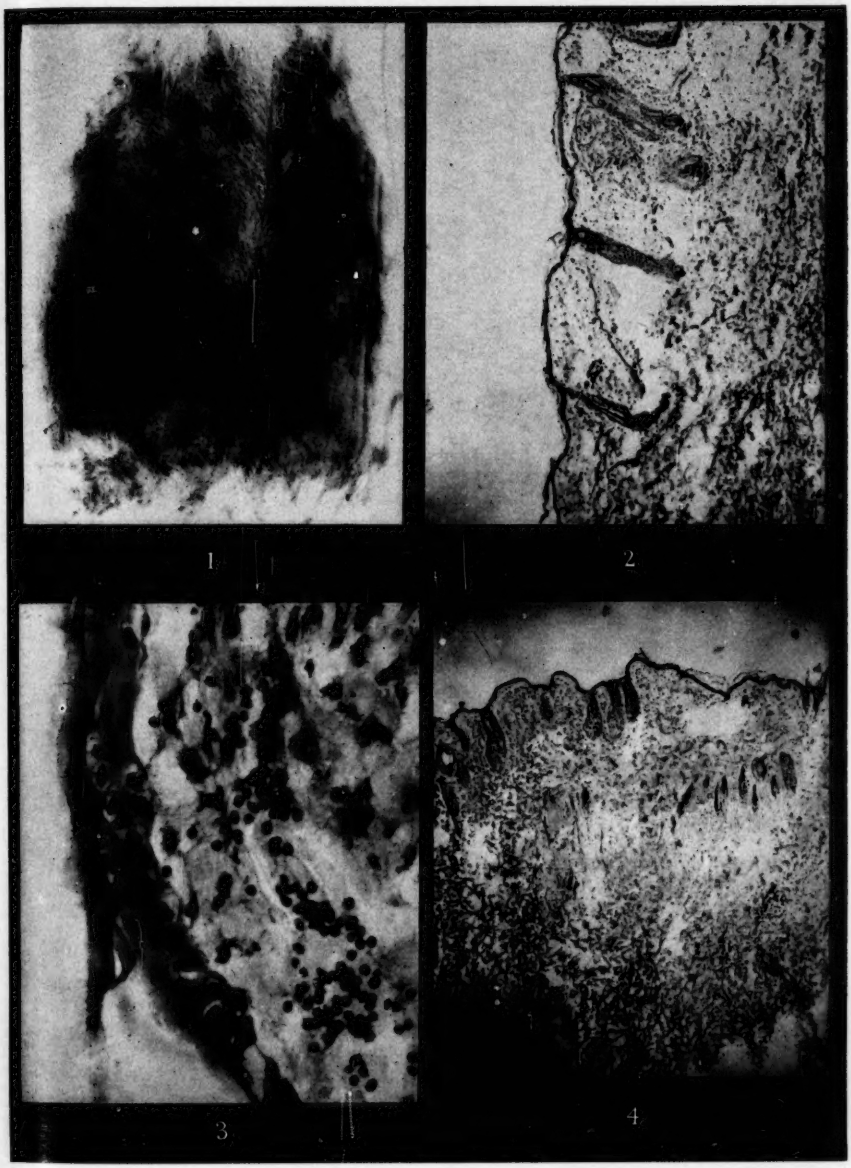
FIG. 4.—Histological appearance of reaction at skin site prepared with sonically vibrated PR8 virus suspension 5 hr. after the intravenous injection of culture filtrate of *Esch. coli* (Rabbit 9). ×36.

FIG. 5.—Higher magnification of the section shown in Fig. 4. The upper layer of the skin. ×128.

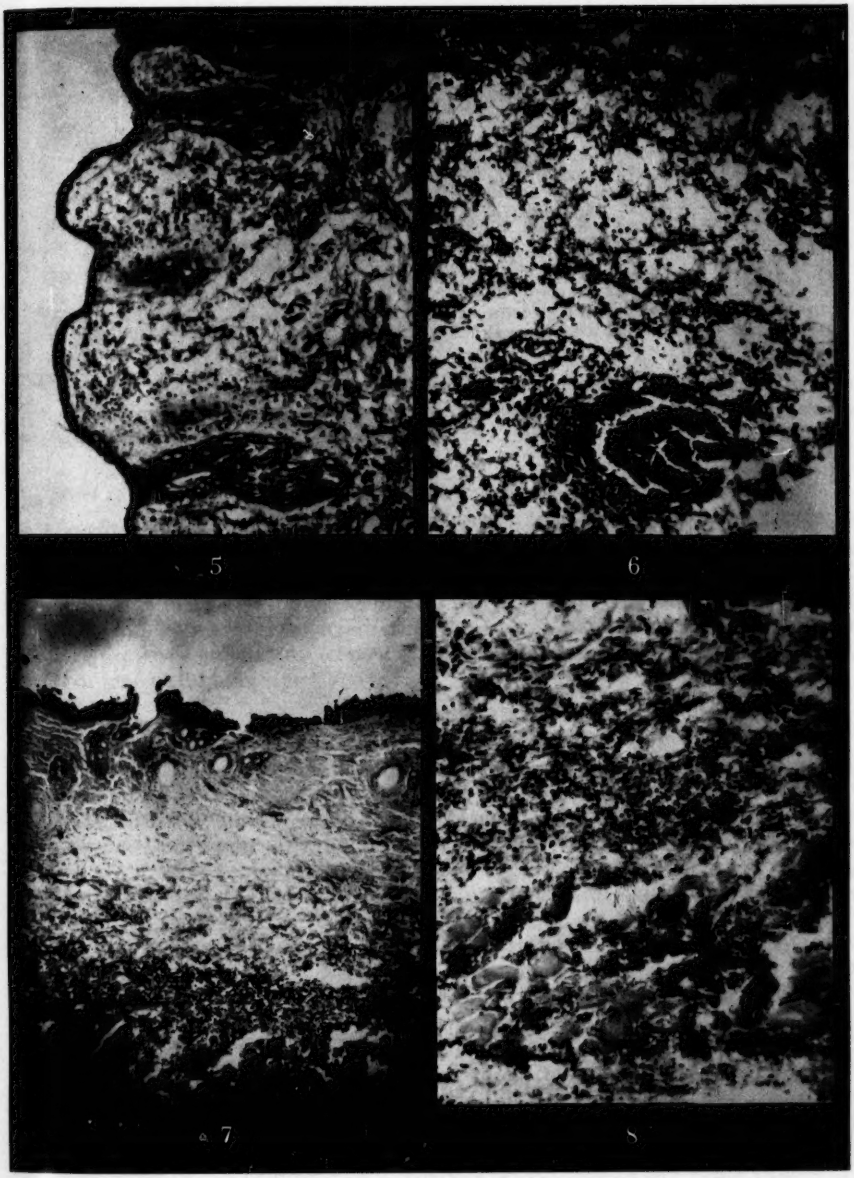
FIG. 6.—Higher magnification of the section shown in Fig. 4. The lower layer of the skin. ×128.

FIG. 7.—Histological appearance of reaction at skin site prepared with sonically vibrated PR8 virus suspension 5 hr. after the intravenous injection of PR8 virus suspension (Rabbit 16). ×48.

FIG. 8.—Higher magnification of the section shown in Fig. 7. The subcutaneous tissues and muscles. ×128.



Kato, Shimizu and Hara.



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Kato, Shimizu and Hara.

TABLE VI.—*The Provocative Potency of Influenza Virus*

Rabbit number	Preparatory injection*	Provocative injection†	Reaction after: Degree	Reaction after: Diameters of haemorrhage (mm,)
16	Sonically vibrated PR8	PR8 suspension	2+	8×8
	Sonically vibrated Lee		2+	6×7
	Sonically vibrated Adachi		2+	8×9
	Coli filtrate		3+	15×18
17	Frozen and thawed PR8	PR8 suspension	2+	7×7
	Frozen and thawed Lee		2+	5×5
	Frozen and thawed Adachi		2+	8×9
	Coli filtrate		3+	15×15

* HA titres of all the virus suspensions before treatments were 10,240 per ml.
† Dose of the provocative injection was 5,120 × 2·5 HA units per kg. of body weight.

The gross appearances of the haemorrhagic reactions provoked by the virus were quite similar to those provoked by culture filtrate of *Esch. coli*. The process of healing was also similar in the two cases.

The effect of some inactivating agents on the active principle of influenza virus

The effects of heat, ultraviolet irradiation and sodium ethylmercurithiosalicylate (Merzonin) on the skin-preparatory and provocative potencies of PR8 virus were examined. The results are summarized in Table VII. It is seen that both potencies were inactivated by heating at 56° for 30 min., treatment with merzonin 1/10,000, and by ultra-violet irradiation for 10 min. After these treatments the infective property was destroyed nearly completely whereas the haemagglutinating property was well maintained.

Histological appearances in the Shwartzman phenomenon induced by influenza virus

Intradermal injections of influenza virus suspensions and culture filtrate of *Esch. coli* could cause erythema and oedema at the injected skin sites. However, intradermal injection alone could not bring about petechial reactions. Erythema and oedema were more obvious at the sites where virus was injected in higher dose or after treatment with sonic vibration or repeated freezing and thawing. Twenty hours after the intradermal injection (just before the provocative injection) the skin sites injected respectively with the intact PR8 virus, sonically vibrated (1 hr.) virus, frozen and thawed (10 cycles) virus suspensions and with culture filtrate of *Esch. coli* were resected for histological examination. There existed only a quantitative difference between the intensity of the histological changes in the skin sites injected with these preparations. Figs. 2 and 3 show a section of a skin site 20 hr. after the intradermal injection of PR8 virus suspension treated with sonic vibration for 1 hr. (20,480 HA/ml., $10^{6.1}$ EID_{50}/ml.). There is an infiltration of neutrophils in the corium of the skin (Fig. 2) and also a migration of a smaller number of larger mononuclear cells, lymphocytes, eosinophils and plasma cells. Some dilatation of the blood capillaries and oedema are seen in the papillary layer of the skin (Fig. 3). There is no rupture of the blood vessels and little extravasation of erythrocytes. There are no thrombi. Some epithelial cells show swelling and vacuolization (Fig. 3). Histologically the skin site injected with

TABLE VII.—*Effect of Some Inactivating Agents on the Skin-preparatory and Provocative Potencies of Influenza Virus*

Rabbit number	Preparatory injection: Material	HA/ml.	EID_{50}/ml.	Provocative injection	Reaction after provocative injection: Degree	Diameters of haemorrhage (mm.)
20	Sonically vibrated PR8*	20,480	6·2	Coli filtrate	3+	10×10
	Frozen and thawed PR8†	10,240	10·1		2+	7×8
	Sonically vibrated PR8 heated at 56° for 30 min.	5,120	<2		—	—
	Frozen and thawed PR8 heated at 56° for 30 min.	5,120	<2		—	—
	Coli filtrate	..	..		3+	25×25
12	Sonically vibrated PR8	40,960	6·5	Coli filtrate	3+	17×18
	Sonically vibrated PR8 heated at 56° for 30 min.	20,480	<2		1+	—
	Sonically vibrated PR8 heated at 70° for 30 min.	<4	<2		—	—
	Coli filtrate	..	..		3+	30×32
22	Sonically vibrated PR8	20,480	6·2	Coli filtrate	3+	11×11
	Sonically vibrated PR8 UV-irradiated‡	20,480	<2		—	—
	Sonically vibrated PR8 treated with Merzonin§	20,480	<2		—	—
	Coli filtrate	..	..		3+	22×23
23	Sonically vibrated PR8	40,960	6·5	PR8‖ heated 56° 30 min.	—	—
	Frozen and thawed PR8	20,480	10·4		—	—
	Coli filtrate	..	..		—	—
24	Sonically vibrated PR8	40,480	6·5	PR8‖ UV-irradiated	—	—
	Frozen and thawed PR8	20,480	10·4		—	—
	Coli filtrate	..	..		—	—
25	Sonically vibrated PR8	40,960	6·5	PR8‖ treated with Merzonin	—	—
	Frozen and thawed PR8	20,480	10·4		—	—
	Coli filtrate	..	..		—	—

* Sonic vibration for 1 hr.

† Freezing and thawing were repeated 10 times.

‡ Ultraviolet irradiation was carried out at a distance of 30 cm. for 10 min. using a germicidal lamp (Mitsubishi Electric Co.).

§ Treated with sodium ethylmercurithiosalicylate (Merzonin, Takeda Co.) 1 : 10,000 at 37° for 4 hr. and at 4° overnight.

‖ Titres of the PR8 virus suspension before treatments were 20,480 HA/ml. and $10^{10.5}EID_{50}$/ml. Dose injected was 2·5 ml. per kg. of body weight.

culture filtrate of *Esch. coli* was qualitatively all the same as the site injected with sonically vibrated virus except for the changes in the epithelical cells.

Sections of the haemorrhagic areas of skin sites prepared with sonically vibrated virus suspension (20,480 HA/ml., $10^{6.1}$ EID_{50}/ml.) and with culture filtrate of *Esch. coli* were obtained 5 hr. after the provocative injection of culture filtrate of *Esch. coli* (Rabbit 9) and after the provocative injection of PR8 virus suspension (Rabbit 16). Histological appearances of the haemorrhagic reactions in these skin sites were qualitatively all the same irrespective of the kind of preparatory and provocative materials employed. Figs. 4–8 show sections of the haemorrhagic areas of the skin obtained from Rabbit 9 (Figs. 4, 5 and 6) and

Rabbit 16 (Figs. 7 and 8). There are extensive haemorrhages in the papillary layer and upper corium of the skin (Figs. 5 and 7). The haemorrhage extends to the lower layer of the corium, subcutaneous tissues and to the muscle layer (Fig. 8). There is also an extensive migration of neutrophils in the corium, especially in its lower layer (Figs. 6 and 8). Migration of a small number of large mononuclear cells, eosinophils, lymphocytes and plasma cells can also be seen. A number of the migrated cells are degenerated or necrotic, showing fragmentation and pyknosis of the nuclei. There is thrombosis of the small veins in places in the corium (Fig. 6). The endothelial cells of these small veins show hyperplasia, swelling and desquamation.

DISCUSSION

In the studies reported here, it was found that influenza virus particles possessed both the preparatory and provocative factors of the Shwartzman reaction. These factors could not be separated from the virus particles by such means as high-speed centrifugation and adsorption onto and elution from red cells. Both these factors were readily inactivated by heat, ultraviolet irradiation and by sodium ethylmercurithiosalicylate. Their stability against these agents was similar to that of the toxic agent reported in previous papers (Kato and Hara, 1961; Kato and Okada, 1961). Furthermore, the preparatory factor was similar to the toxic agent in the following respects. The activity was strengthened by sonic vibration in spite of the loss of most of the infectivity. Some of the activity was released from the HA particles by sonic vibration. In other words, the activity could be demonstrated independently of the infective and haemagglutinating activities. Ten cycles of freezing and thawing, which did not cause disintegration of the virus particles, could uncover the preparatory potency to the same extent as 1 hr.'s sonic vibration, which disintegrated the virus particles nearly completely into subunits (Kato and Okada, 1961). From these data it can be assumed that most of the preparatory factor is located near the surface of the virus particle. It has already been indicated that most of the toxic agent is located near the surface of the virus particle (Kato and Okada, 1961).

In the skin sites injected with influenza virus no virus multiplication took place as determined by haemagglutination and infectivity titrations. It is, therefore, reasonable to regard the phenomenon of local skin reactivity induced by the intradermal injection of influenza virus as a type of virus-induced toxic reaction. It is most likely that the active principle of the phenomenon associated with influenza virus particles is identical in nature with the toxic agent described in previous papers (Kato and Hara, 1961 ; Kato and Okada, 1961).

Haemorrhagic reactions in the skin sites prepared with influenza virus could also be elicited by the provocative injection of bacterial active principle derived from *Esch. coli* culture. The histological appearances of the haemorrhagic areas were qualitatively the same in the following 4 combinations of preparatory and provocative materials : sonically vibrated PR8 virus suspension (preparatory) and PR8 virus suspension (provocative) ; sonically vibrated PR8 virus suspension and culture filtrate of *Esch. coli* ; culture filtrate of *Esch. coli* and PR8 virus suspension ; culture filtrate of *Esch. coli* and culture filtrate of *Esch. coli*. The histological findings were essentially identical with those described in detail by Shwartzman (1937), working with the phenomenon of local tissue reactivity induced by bacterial active principles. The conspicuous histological changes were

extensive haemorrhage, infiltration of neutrophils and venous thrombi. It seems to be probable that the preparatory factor acts more or less directly upon the endothelial cells of the blood capillaries and small blood vessels in such a way as to yield a preparatory state for the rupture of the blood vessels and production of the venous thrombi. On the other hand, the mechanism of an extensive infiltration of neutrophils cannot be fully understood. In general, viral inflammation is rarely accompanied with an infiltration of this type of leucocyte (Burnet, 1955).

The active principle associated with influenza virus particles was heat-labile in contrast with the heat-stability of the bacterial active principles (Shwartzman, 1937). The intradermal injection, alone, of influenza virus suspension into rabbits could create an area of mild inflammation of the skin but rarely cause petechial reactions as already described by Shimizu (1959). The ability to evoke skin inflammation seems to be related to the skin-preparatory potency. It was, however, known that the ability of other active principles to evoke inflammation was not necessarily accompanied by the skin-preparatory potency (Shwartzman, 1937).

SUMMARY

It was found that influenza virus particles possessed both the skin-preparatory factor, capable of inducing local skin reactivity when given by intradermal injection into rabbits, and the provocative factor which elicited, when given by intravenous injection, a haemorrhagic reaction at the prepared skin sites. The skin-preparatory potency was uncovered by sonic vibration and repeated freezing and thawing. The active principle of the Shwartzman phenomenon associated with influenza virus was similar in some respects to its toxic agent which caused death of experimental animals with characteristic lesions.

Virus multiplication did not take place in the skin sites injected with influenza virus as determined by haemagglutination and infectivity titrations. It can, therefore, be assumed that the phenomenon of local skin reactivity induced by influenza virus is a type of virus-induced toxic reaction.

The histological appearances of the haemorrhagic areas were qualitatively the same irrespective of whether the active principles employed as the preparatory and provocative materials were derived from influenza virus or *Esch. coli*.

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CEREBRAL VASCULAR DAMAGE IN GUINEA-PIGS INDUCED BY VARIOUS HETEROPHILE ANTISERA INJECTED BY THE FORSSMAN INTRACAROTID TECHNIQUE

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THE "carotid syndrome" was discovered accidentally by Forssman (1920) during his analysis of the mechanism of the anaphylactoid shock precipitated in guinea-pigs by an intravenous injection of rabbit anti-sheep red cell serum. At the time, he was trying to eliminate any participation by the brain, heart and lungs in the pathogenesis of the reaction, and to concentrate on any possible contribution made by the abdominal organs. By injecting the antiserum centripetally into the right common carotid artery, he intended to introduce it first into the arch and later into the abdominal aorta, but as can be seen from the anatomical diagram (Fig. 1), an unsuspected route was available for some of the antiserum to be swept by the flowing blood first into the right subclavian artery and thence into the right vertebral artery. As a result of this diversion of the antiserum to the brain, many of his guinea-pigs died promptly with a characteristic syndrome indicative of serious injury to important motor nerve nuclei in the medulla oblongata.

Forssman's carotid syndrome has since been studied by several investigators (Skoog, 1939). The lesions acquired an added interest, however, when Jervis (1943) observed that in those animals that survived the syndrome for several days, scattered areas of demyelination appeared in the brain stem, though since many of the axons had been lost, this may have been mainly of a secondary type. The condition thus provides an interesting form of allergic injury to the central nervous system in which the antigenic component of the reaction is a normal constituent of the tissues of the susceptible animal.

In the present study, we have extended these observations, first, by estimating more precisely the severity of such lesions by measuring the escape of radioiodinated homologous serum proteins into selected parts of the damaged brain, and second, by showing that the syndrome can be also provoked by the injection of other types of Forssman antibodies, notably that produced by the immunization of rabbits with a strain of pneumococcus that contains this heterophile antigen. Such a form of allergic encephalomyelitis would seem to be less remote pathogenetically from a conceivable naturally occurring lesion in man than those forms that follow inoculation with mixtures of emulsified brain and Freund adjuvant.

MATERIALS AND METHODS

Animals.—Albino guinea-pigs weighing from 250–400 g. and of both sexes were used. The rabbits employed for producing the immune serums were all tested for their initial sheep red cell haemolysin titres; none was greater than 1/10.

Forssman antisera.—The "Forssman antigen" used in this study is defined as one that when injected into rabbits produces a haemolysin for sheep red cells which can be absorbed by guinea-pig kidney. Three different antiserums of this type (anti-sheep red cells, anti-dog lung and anti-pneumococcus) were employed; all fulfilled the above criteria.

Anti-sheep red cell serum.—These were prepared by injecting 2 ml. of a 10 per cent suspension of sheep red cells in saline intravenously at weekly intervals for periods of 6–8 weeks. The rabbits were bled 5 days after the last inoculation. A pooled serum with a haemolysin titre of 1/2048 was used in these experiments. Such a serum contains at least two components, an isophile antibody and one directed against the Forssman or heterophile antigen.

Anti-dog lung serum.—Dog lung was removed aseptically at post mortem; ten grams were minced, homogenized first with saline in a Waring blendor and later, to effect finer

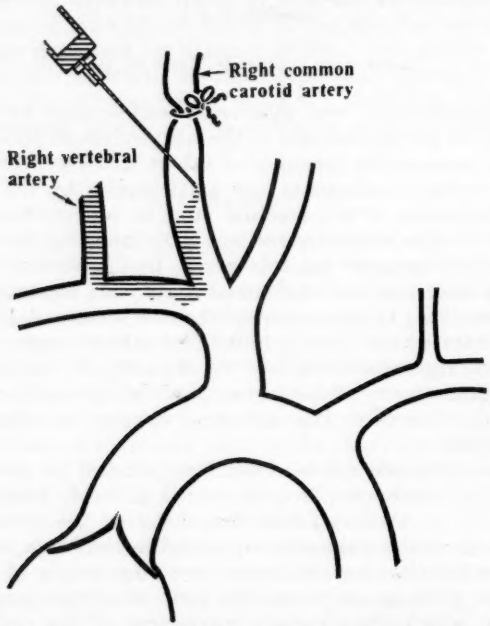


FIG. 1.—Diagram showing the main arteries in the neck of guinea-pigs and the route (indicated by hatching) taken by material injected by Forssman's technique.

triturition, in a glass tissue grinder. The volume of the suspension was made up to 50 ml. with normal saline, and 500,000 units of penicillin and 0·5 g. of streptomycin added. The mixture was filtered through a gauze filter of the type used for human blood transfusion. Rabbits were injected intraperitoneally with 1 ml. of this suspension at weekly intervals for 5 weeks. They were bled 5 days after the last injection. The serum used had a haemolytic titre of 1/1024.

Anti-pneumococcus serum.—A Griffith rough strain of the organism (R36NC) was used as antigen. It was grown for 18 hr. on glucose broth, killed by heating to 60°, centrifuged and re-suspended in normal saline to a concentration of 1×10^{10} organisms per ml. Rabbits were injected intravenously with 1 ml. of this suspension daily for six days; they were bled 5 days after the last inoculation. Marked individual variations in the haemolysin response of rabbits immunized with rough strains of pneumococcus have been reported by Bailey and Shorb (1933) and by Goebel and Adams (1943). Potent serums are difficult to produce with this antigen, for prolonged courses of inoculation generally reduce the haemolysin titre. The rabbits we used, however, were subjected to successive courses of immunization, and the

serum employed in the present experiments was obtained from an animal after its third course of injections: it had a titre of 1/2048.

Haemolysin titres.—The sheep cell haemolysin titres were estimated by the method used by Tanaka and Leduc (1956).

Homologous ^{131}I-labelled serum.—Blood was drawn aseptically by cardiac puncture from large donor guinea-pigs. The separated serum was labelled with ^{131}I by the method of Veall and Vetter (1958).

Operative procedures.—One ml. of the labelled serum was injected into the saphenous vein of the guinea-pig under Nembutal and light ether anaesthesia about 1 hr. before the intracarotid injection of the control or heterophile rabbit serum. To determine the level of the circulating labelled protein, 0·02 ml. of blood was collected from an ear vein just before the intracarotid injection, and added to 10 ml. of normal saline in a universal screw-capped container. The carotid syndrome was produced by the centripetal injection of the Forssman serum into the ligated right common carotid artery under light Nembutal and ether anaesthesia by the method described by Forssman. When possible, the injection volume and injection time were standardized at 0·3 ml. and 15 sec. respectively. The guinea-pigs which survived the immediate phase of the reaction were mostly killed by an overdose of Nembutal from 1–3 hr. later.

Subsequent examination of tissues.—The brain was removed, washed with normal saline to remove any surface blood, and fixed for 18 hr. in 10 per cent formol-saline. The fixed brain was further freed from any traces of adherent blood, and divided into 4 portions by a sagittal incision and a transverse cut at the level of the upper end of the pons. This separated the hind brain, together with the cerebellum, from the fore- and midbrains. In some animals, the spinal cord also was removed after fixation *in situ* and divided into 4 equal lengths. The portions of the brain and spinal cord were then weighed and placed in universal screw-capped bottles containing 10 ml. of formol-saline in readiness for their radioactivity to be determined and preparatory to histological examination.

Determination of radioactivity.—All specimens were measured in a "Coronet" multitube gamma counter of the type described by Veall and Baptista (1954).

"*Blood equivalent*".—In order to achieve comparability between the various animals used, the tissue radioactivity was in each case related to the corresponding level of blood radioactivity. By dividing the c.p.m./g. for each specimen by the c.p.m./ml. for the blood of the respective animal, an index, the "blood equivalent" was derived. In this way allowance was made for the individual differences in the blood radioactivity of the various guinea-pigs. This index, expressed as a percentage value, provides a comparative measure of injury inflicted by the various serums on the blood vessels in different parts of the brain.

Histology.—At least 4 blocks were taken from each brain; they were from the fore-brain at the level of the optic chiasma, the midbrain, the pons and cerebellum and the medulla oblongata. Complete transverse sections were stained with haematoxylin and eosin and with cresyl violet. With some animals, additional sections were stained by Loyez's method for myelin and by that of Marsland, Erikson and Glees (1954) for axons.

RESULTS

Estimation of the Extent of Vascular Injury in Different Portions of the Central Nervous System with the Aid of Circulating Labelled Proteins

The Brain

Control serum.—When normal rabbit serum was injected by Forssman's method into guinea-pigs in which labelled serum proteins were circulating, the radioactivities of the 4 portions into which the brain was later divided differed little from one another. The mean values (Table I) of the blood equivalents of the left and right fore brains were $1{\cdot}29 \pm 0{\cdot}19$ and $1{\cdot}33 \pm 0{\cdot}22$ and those of the corresponding hind brains were $1{\cdot}74 \pm 0{\cdot}25$ and $1{\cdot}72 \pm 0{\cdot}28$ respectively. These values show that in spite of ligation of, and injection of foreign serum into, the right common carotid artery, there was no preferential accumulation of the labelled protein in the right half of the brain. While the hind brain as a whole had a fractionally

TABLE I.—*" Blood Equivalents " in the Brains of Guinea-pigs Injected by the Carotid Technique with Normal Rabbit Serum*

Dose of control serum (ml.)	Duration of experiment (hr.)*	Blood equivalents (per cent)			
		Left fore- + midbrain	Right fore- + midbrain	Left hind	Right hind
0·05	K2	1·16	1·17	1·54	2·05
0·05	K3	1·46	1·16	2·01	1·53
0·05	K2	2·25	2·44	2·51	2·84
0·05	K2	1·39	1·65	2·43	2·41
0·075	K2	1·11	1·28	1·85	1·22
0·70	K2	0·78	0·69	0·91	0·86
0·90	K2	0·87	0·92	0·92	1·12

* K = killed.

higher blood equivalent than the fore brain, in no instance did the radioactivity in any portion of these control brains reach a blood equivalent of three per cent.

Anti-sheep red cell serum.—In guinea-pigs injected with anti-sheep red cell serum, the radioactivity of certain portions of the brain rose significantly. The chief increase took place in the right hind brain, while sometimes the right fore and sometimes the left hind brain came next in order. Almost invariably, the left fore brain proved to be the least radioactive of the 4 portions, and was never found to rise above the upper limit found in the control animals (Table II).

TABLE II.—*" Blood Equivalents " in the Brains of Guinea-pigs Injected by the Carotid Technique with Rabbit Anti-sheep Red Cell Serum*

Dose of Forssman serum (ml.)	Duration of experiment (hr.)*	Blood equivalents (per cent)			
		Left fore- + midbrain	Right fore- + midbrain	Left hind	Right hind
0·003	K 2	1·49	1·51	1·97	2·29
0·010	D 1¾	2·40	2·54	5·05	10·70
0·03	K 2	1·66	2·81	4·47	7·88
0·05	K 2	1·61	1·54	2·21	2·33
0·05	K 1	1·4	4·4	2·4	4·5
0·05	K 3	1·0	2·0	3·0	6·5
0·05	D10 min.	1·51	4·38	2·53	7·97
0·05	K 2	2·11	5·30	4·53	10·2
0·05	D30 min.	2·11	11·80	4·28	12·95
0·10	D15 min.	2·25	11·98	3·02	14·62

* D = died. K = killed.

A more detailed examination of the site of the vascular injury in the brains of 2 animals showed that the cerebellum is little affected in the carotid syndrome (Table III); this finding agrees with comparable observations made by Broman (1938) who used trypan blue as an indicator of local damage.

TABLE III.—*" Blood Equivalents " in Different Parts of the Brain in Guinea-Pigs Injected by the Carotid Technique with Rabbit Anti-sheep Red Cell Serum*

Cerebellum		Pons and medulla		Midbrain		Cerebral hemispheres	
L.	R.	L.	R.	L.	R.	L.	R.
1·57	3·15	8·48	16·00	1·12	8·70	2·07	4·55
1·71	1·48	6·59	20·10	0·50	7·69	1·44	12·35

To obtain some idea of the time course of the vascular injuries, 4 guinea-pigs were injected with a sublethal dose (0·0125 ml.) of the antiserum and killed at intervals subsequently. Signs of damage were apparent at the end of 30 min., seemed to reach a maximum between 2 and 6 hr. and were no longer appreciable at 24 hr. (Table IV).

TABLE IV.—*"Blood Equivalents" in the Brains of Guinea-pigs at Various Times After the Injection by the Carotid Technique of Rabbit Anti-sheep Red Cell Serum*

Duration of experiment	Blood equivalents (per cent)			
	Left fore- + midbrain	Right fore- + midbrain	Left hind	Right hind
30 min.	1·76	1·71	2·68	5·67
2 hr.	2·00	5·27	3·02	9·75
6 hr.	1·30	2·38	3·09	12·11
24 hr.	2·00	2·07	2·69	2·52

Anti-pneumococcus serum.—Eight guinea-pigs were given anti-pneumococcus serum in various dosages. In amounts of 0·1 ml. or less, there was no evidence of vascular injury in any of the 4 portions of the brain (Table V). But in 4 of the 5 animals given serum in excess of this quantity, the radioactivity of the hind brain on the right side became substantially increased. With the higher doses used, there was also evidence that the injury had extended into the right fore brain and, to a lesser extent, into the left hind brain as well.

TABLE V.—*"Blood Equivalents" in the Brains of Guinea-pigs Injected by the Carotid Technique with Rabbit anti-Pneumococcus Serum.*

Dose of Forssman serum (ml.)	Duration of experiment (hr.)*	Blood equivalents (per cent)			
		Left fore- + midbrain	Right fore- + midbrain	Left hind	Right hind
0·04	K 2	2·08	2·19	2·25	2·29
0·10	K 2	1·47	1·45	1·45	1·27
0·10	D 20 min.	1·17	1·30	1·22	1·81
0·20	K 2	1·20	2·67	2·58	5·83
0·30	K 2	1·24	1·19	1·20	2·02
0·60	K 2	1·90	12·77	5·84	16·45
0·85	K 2	2·13	8·05	2·38	5·14
0·90	D 1½	3·53	17·65	6·37	24·30

* D = died. K = killed.

Anti-dog lung serum.—Three guinea-pigs were injected with this type of Forssman antiserum in ascending doses and with comparably increasing signs of injury. With the largest inoculum given, death took place after 15 min. with a marked accumulation of radioactive protein in the brain which had a pattern of distribution similar to that found with the other types of Forssman antiserum (Table VI).

The spinal cord

Since in the guinea-pig the median artery of the spinal cord is joined at its upper end by branches from the vertebral arteries (Knox-Macauley, Morrell, Potts and Preston, 1960), it seemed possible that some of the injected antiserum might

TABLE VI.—*" Blood Equivalents " in the Brains of Guinea-pigs Injected by the Carotid Technique with Rabbit Anti-dog Lung Serum*

Dose of Forssman serum (ml.)	Duration of experiment (hr.)*	Blood equivalents (per cent)			
		Left fore- + midbrain	Right fore- + midbrain	Left hind	Right hind
0·25	K 2	1·17	0·98	1·51	1·08
0·50	D 1½	2·85	3·13	5·80	15·70
1·00	D 15 min.	3·82	12·59	7·25	23·72

* D = died. K = killed.

have been carried into its upper segments. For this reason, the radioactivity of the upper quarter of the spinal cord was measured. In 3 normal animals, the mean blood equivalent of this part of the cord was 2·66, while in 7 that had been given anti-sheep red cell serum and in 5 that had received the larger doses of anti-pneumococcus serum, the values were 7·57 and 6·03 respectively.

Histological changes produced in the brain by Forssman antisera

Our microscopical findings in the affected portions of the central nervous system agree well with those of Jervis. The earliest change is a widespread dilatation of the small vessels in the pons and medulla oblongata (Fig. 2), which in some animals extends to the midbrain and the cerebral hemispheres on the injected side. The lesion is almost wholly unilateral and the midline is often clearly demarcated. Although the brachium pontis is frequently affected, no lesions have been seen in the cerebellum. When the dose of antiserum is large, scattered haemorrhagic foci are present in addition to the vasodilatation. These small haemorrhages are especially numerous in animals given anti-pneumococcus serum (Fig. 3).

In guinea-pigs that died 6–12 hr. after the injection, large circumscribed areas of oedema (Fig. 4) with grave degenerative changes in both neurones and neuroglial cells, are conspicuous in the affected regions (Fig. 5). These parenchymatous lesions appear to be secondary to the damage to the small blood vessels ; such a sequence of changes might be anticipated from the known distribution of the Forssman antigen in the endothelium of the blood capillaries in the brain.

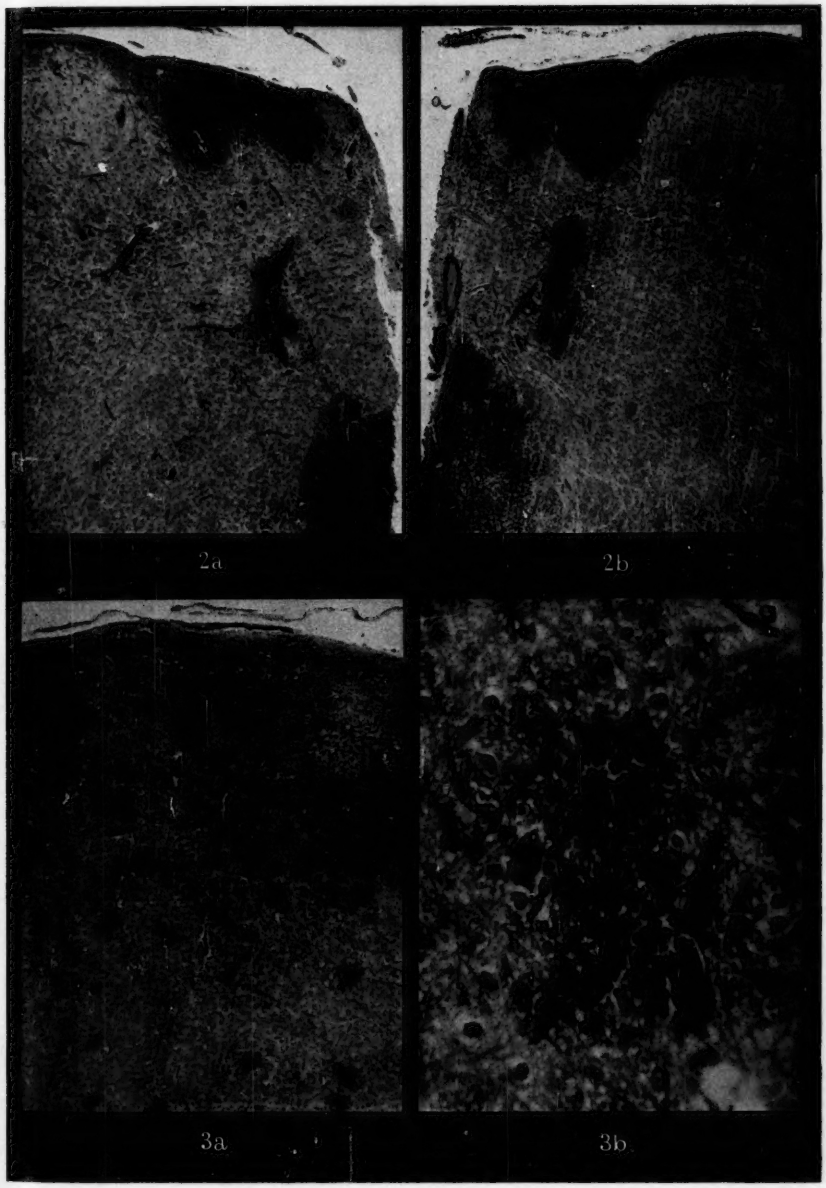
EXPLANATION OF PLATES.

FIG. 2.—Transverse sections from the medulla oblongata of a guinea-pig injected with anti-sheep red cell serum showing hyperaemia on the right side : (a) control (left) side ; (b) injected (right) side. H. and E. ×32.

FIG. 3.—Transverse section from the right side of the medulla oblongata of a guinea-pig injected with anti-pneumococcus serum 2 hr. previously, showing widely distributed small haemorrhages : (a) ×32 ; (b) ×360. H. and E.

FIG. 4.—Transverse section through the medulla oblongata of a guinea-pig injected with anti-sheep red cell serum and killed after 6 hr. showing the swollen and oedematous areas on the right side. H. and E. ×16.

FIG. 5.—Higher magnification of sections from the medulla oblongata shown in Fig. 4 displaying the degenerative changes in the neurones and neuroglial cells together with the oedema : (a) control side ; (b) injected side. ×360.



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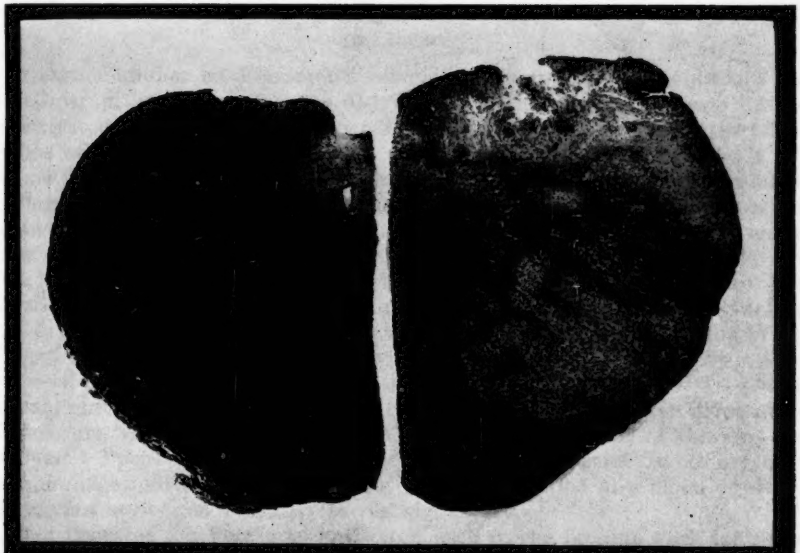

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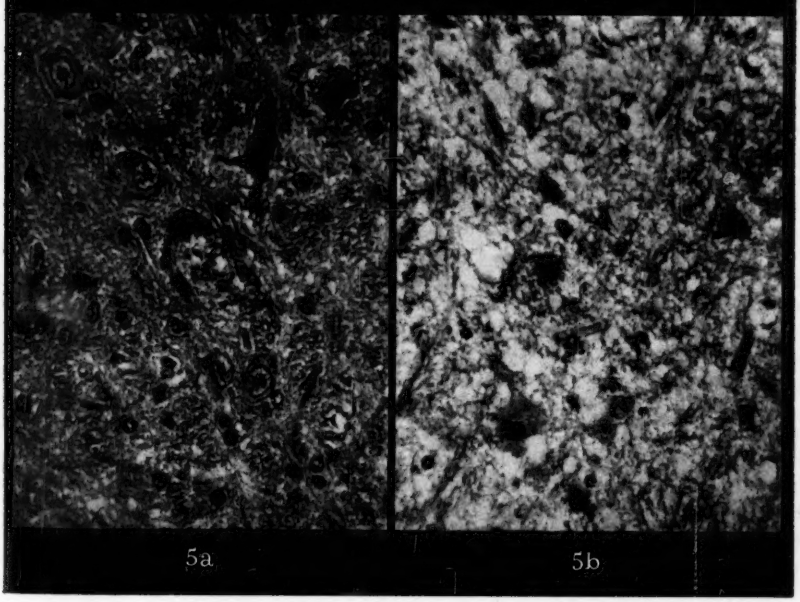
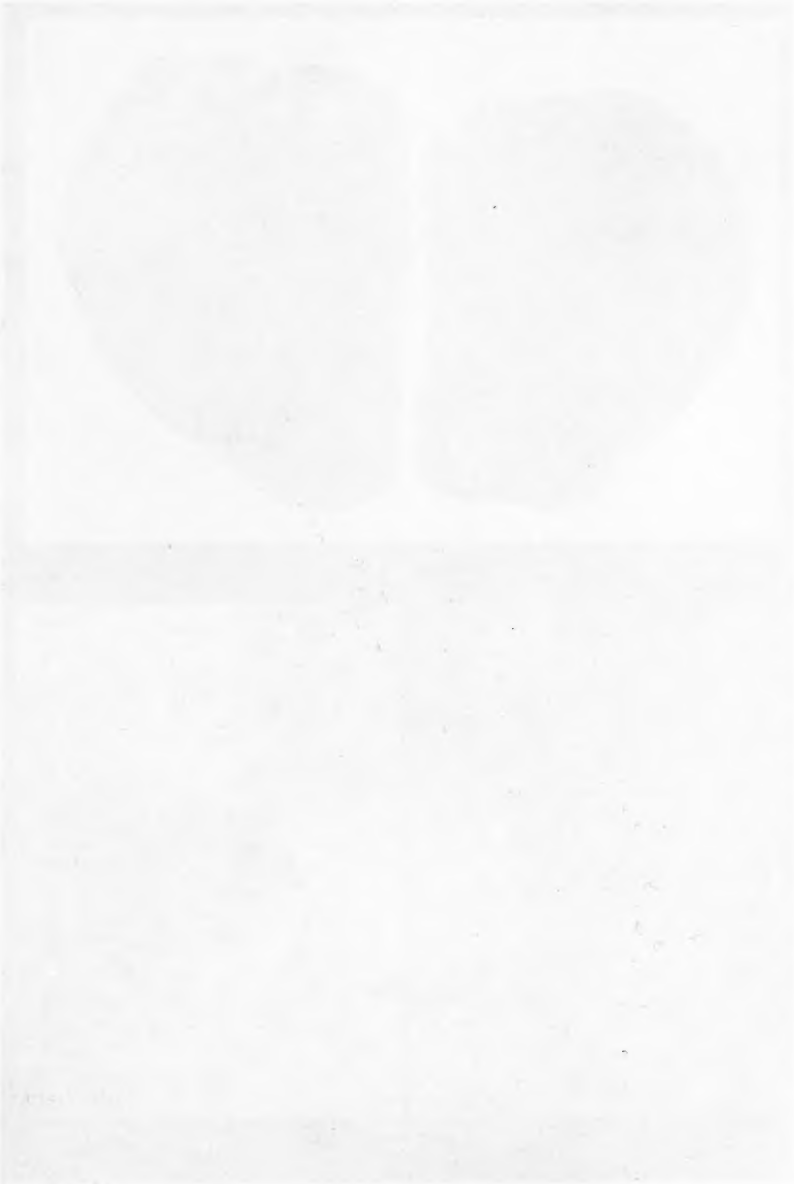


5a 5b



DISCUSSION

In earlier studies on Forssman's carotid syndrome, in which only anti-sheep red cell serum has been used, the distribution of the vascular damage has been demonstrated by the escape of trypan blue and other vital dyes into the affected portions of the brain (Broman, 1938 ; Jervis, 1943). These dye methods, however, are little better than qualitative, and their replacement by circulating labelled homologous serum proteins allows a quantitative estimate of the extravasation of plasma or blood at any sites of damage. In studies on the normal central nervous system, in which radioactive plasma proteins have been introduced into the circulation, the proportion of the excised brain that is occupied by blood (the " blood equivalent ") has been found to vary little among the small laboratory animals. In rabbits it is about 1·3 per cent (Boyse, Morgan, Pearson and Payling Wright, 1956) ; in exsanguinated rats it is about 1·1 per cent (Dewey, 1959) ; and in fowls used as controls in other carotid syndrome studies it is about 1·1 per cent (personal observations). In our control guinea-pigs, which had been given normal rabbit serum by the Forssman method, the blood equivalent of the fore brain was about 1·3 per cent and of the hind brain about 1·7 per cent. In our evaluation of the damage inflicted by the antisera, we have regarded any blood equivalent of 3 per cent or over as indicative of vascular injury.

All 3 types of the Forssman antiserum used in the present work (anti-sheep red cell, anti-dog lung and anti-pneumococcus) were able to produce the characteristic fatal carotid syndrome, though their relative potencies, as judged by the volume of antiserum needed, varied. Further, in all the animals in which the condition was evoked, the distribution of the extravasated labelled protein followed the pattern imposed by the anatomical distribution of the cerebral arteries.

The histological changes that develop in the brain in this syndrome have been described previously (Friedberger and Schröder, 1922 ; Ingvar, 1927 ; Skoog, 1939 ; Jervis, 1943), and only such amplification as seems relevant to the present work will be discussed here. Our findings suggest that the brunt of the injury falls on the vascular endothelium, which might be expected from the observation of Tanaka and Leduc (1956), using fluorescein-labelled antibody, that the Forssman antigen is concentrated in these cells. A feature of interest that emerges from the present work, however, is that signs of raised vascular permeability, as disclosed by an elevated radioactivity of the affected brain substance, and presumably a sign of incipient injury, appear before histological evidence of damage becomes recognizable. In 13 animals with an increase in the radioactivity of the right hind brain, only 9 showed any morphological changes. For the right fore brain and the left hind brain, the difference was more notable—the figures being 4 out of 11 and 0 out of 11 respectively. The accumulation of the labelled protein in such brains could be accounted for either by vasodilatation and haemorrhage, or by an extravasated exudate. In those experiments in which large doses of antiserum were given, all 3 features were often present. But since in most animals, large increases in radioactivity were recorded in the absence of histological evidence of either vasodilatation or haemorrhage, they would seem to be attributable mainly or wholly to the escape of exudate.

Previously, the carotid syndrome has been obtained only with heterophile sera prepared against sheep red cells. The present study has extended these observations by demonstrating that similar injuries can be inflicted on the brain

in guinea-pigs by at least 2 other types of Forssman antibody, one made against dog lung (which is known to be rich in this antigen) and the other against a commonly found bacterium, a pneumococcus. Many forms of heterophile reactions have attracted some attention in the past, and their possible relevance to disease processes has been discussed by several authors (Buchbinder, 1935 ; Landsteiner, 1946 ; Dujarric de la Rivière and Eyquem, 1953 ; Kabat, 1956). Among those of particular interest in human pathology are the marked elevation of the α-agglutinin titre in Group O persons that follows inoculation with tetanus antitoxin and various prophylactic agents for whose preparation hog pepsin has been employed (Crawford, Cutbush, Falconer and Mollison, 1952) and the agglutination of human red cells *in vivo*, sometimes with fatal consequences, that has followed the therapeutic use of anti-pneumococcus serum of Type XIV (Finland and Curnen, 1940). Such reactions have in the main been chance findings, and those hitherto recognized are almost certainly only a small proportion of those that exist. The production of a fatal allergic encephalomyelitis in guinea-pigs through the injection of an antibody prepared against a pneumococcus discloses how widely these heterophile reactions can operate as destructive pathogenetic mechanisms.

SUMMARY

The Forssman carotid syndrome has been produced in guinea-pigs with 3 types of heterophile antisera (anti-sheep red cell, anti-dog lung and anti-pneumococcus).

The degree of damage produced by these heterophile antisera has been determined with the aid of previously injected ^{131}I-labelled homologous serum. This method of estimation proved more sensitive than histological examination for the detection of local damage.

The site of injury in the brain is determined by the distribution of the arterial blood supply. The injury, which falls mainly on the vascular endothelium, results in local cerebral oedema and haemorrhage.

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SEROLOGICAL METHODS IN THE STUDY OF TRANSPLANTATION ANTIGENS

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WHEN lymphoid cells or other grafts from A-strain mice are transplanted into mice of strains CBA or C3H, they produce effects of 2 distinct kinds: (a) they excite transplantation immunity, as shown by the abnormally rapid destruction of an A-strain skin graft transplanted a few days later—the so-called "second set" response; and (b) they excite the formation of humoral antibodies, among them haemagglutinins (Gorer, 1937), haemolysins (Hildemann, 1957) and cytotoxins (Gorer and O'Gorman, 1956). The antigens that provoke these 2 responses may or may not be the same, but because they have a common genetic determination they may well have similar immunologically determinant groups (Snell, 1957; Medawar, 1959*a*). If this is so, then antigens that sensitize their recipients to homografts should be capable of absorbing the corresponding humoral antibodies or of inhibiting their action. Thus an antigen prepared from the tissues of A-strain mice which excites transplantation immunity in CBA mice should absorb *in vitro* the haemagglutinins formed by the repeated injection of A-line lymphoid cells into CBA mice. Hildemann and Medawar (1959) found no convincing evidence that this was so, but their antigenic material—often in the form of nuclei or nuclear fragments—was particularly crude, and their failure to absorb humoral antibodies may have been due to the inaccessibility rather than to the absence of the appropriate combining sites. The evidence reported here shows that "transplantation antigens" extracted by the method of Billingham, Brent, and Medawar (1958) are indeed capable of absorbing or inhibiting the action of iso-antibodies causing the agglutination or lysis of A-strain red cells; furthermore, their repeated injection into CBA mice provokes the formation of a high titre of haemagglutinins. This promises to provide a valuable auxiliary method of tracing antigenic activity through various preparative procedures, and ultimately of attempting a chemical identification.

MATERIALS AND METHODS

Mice.—We have used adult mice of strain A as donors, and of strains CBA or (less often) C3H as recipients.

Antigen was made from A-strain lymphoid tissue (spleen, lymph nodes, thymus) by the method of Billingham *et al.* (1958), modified in 2 minor ways: (a) at Stage 2 of their preparation a top-drive M.S.E. macerator was substituted for a blender of the piston and cylinder type to disperse the gelatinous cellular sediment in water: treatment for 2 min. sufficed, and the time of exposure to ultrasound was reduced from 60 to 30 sec.; (b) at Stage 4, antigenic matter was spun down at 30,000 **g** instead of at 25,000 **g**. All absorption tests made use of the antigenic sediment produced by this final centrifugation, but the inhibition test made use of the "soluble" preparation (Stage 3) as it exists immediately prior to the final

centrifugation. Great pains were taken to standardize the preparation of antigen, particularly with respect to procedures affecting the degree of dispersion of the antigenic particles, and to make sure that the losses of substance at any stage were reduced to the very minimum. For want of a better method, one unit of antigenic matter is described as the quantity extracted from 1 mg. wet weight of lymphoid tissue. Four determinations gave 9·9, 9·2, 9·6 and 10·4 μg. as the dry weight of one unit of antigenic sediment. Antigenic preparations were tested for their power to sensitize *in vivo* by the method of Billingham *et al.* (1956, 1958).

Humoral antibodies were produced by the weekly intraperitoneal injection into CBA mice of the living cells (2–2·5 $\times$ 10^7 nucleated cells) expressed from 10 mg. wet weight of lymphoid tissue from CBA $\times$ A hybrid mice. The recipients were given 5 injections at weekly intervals, rested for 2 weeks, given a sixth injection, and bled from the heart a week later. Undiluted immune serum was stored in sealed ampoules at $-20°$. Antibodies were titrated as haemagglutinins by the method of Gorer and Mikulska (1954) or, for special purposes, as haemolysins by the method of Hildemann (1957), using absorbed rabbit complement (Owen, 1958). The 3 separate batches of antiserum used in the experiments described here gave titres as follows :—

serum batch	haemagglutinin titre	haemolysin titre
I	800–3200	400–800
II	3200	400–800
III	6400–12,800	1600–3200

Haemagglutination readings were taken by the slide method after 90 min. at 37° ; the degree of haemolysis was read after 60 min. and again after 120 min. at 37°. The haemagglutination-inhibition tests made use of a 3-part system : (i) 0·025 ml. antiserum in falling dilutions in 2 per cent dextran in 0·15 M NaCl ; (ii) 0·025 ml. of 0·15 M NaCl containing the substance suspected of inhibitory power ; and (iii) 0·025 ml. of a 3 per cent suspension of washed A-strain red cells in a heat-inactivated and absorbed human serum diluted with an equal volume of 0·15 M NaCl. Lysis inhibition tests made use of a 4-part system, with " Mg-saline " as diluent throughout (NaCl 0·14 M, sodium phosphate pH 7·6 0·01 M, $MgCl_2$ 0·001 M). The 4 components in order of addition were : (i) 0·05 ml. A-strain red cells washed twice in citrated saline and twice in Mg.-saline, and resuspended in the ratio 0·1 ml. whole blood to 5 ml. Mg.-saline ; (ii) 0·05 ml. of the substance suspected of inhibitory power ; (iii) 0·05 ml. antiserum in falling dilutions ; and, after a 5-min. interval, (iv) 0·05 ml. absorbed rabbit serum diluted by 1 volume to 4.

Absorptions were carried out by mixing various quantities of antigenic sediment redispersed in 0·15 M NaCl with equal volumes of 1/25 (batches I, II) or 1/50 (batch III) antiserum in 2 per cent dextran in 0·15 M NaCl. Absorption was allowed to proceed at room temperature for 20–25 min., and insoluble matter removed by centrifugation at 30,000 **g** for 30 min.

Most of our experiments on absorption or inhibition by antigenic extracts (as opposed to purified chemical substances) were founded on the haemagglutination test. The results of the lytic test were not at variance with those from the haemagglutination tests, but the strongly anticomplementary activity of the antigenic tissue extracts made the lytic technique unsuitable for routine use.

RESULTS

Specificity of absorption and inhibition.

By exposing 1·0 ml. 1/50 antisera I or II to varying quantities of antigenic sediment, it was found that 15 units of A-strain antigen would lower the haemagglutinin titre perceptibly and that 60 units removed all antibodies detectable within the chosen range of dilutions. Under exactly comparable conditions, 500–700 units of " antigen " made from the lymphoid tissues of CBA or C3H mice had no perceptible effect on the haemagglutinin titre. Representative titrations are shown in Table I. (Antiserum III was much more powerful : here, more than 400 units of A-strain antigen were required to exhaust the antibody present in

TABLE I.—*Haemagglutinin Titres After the Absorption, by Various Antigenic Extracts, of Antisera Prepared in CBA Mice Against A-strain Antigen*

Expt. No.	Serum batch	Source of antigen	Units of antigen per 1·0 ml. 1/50 antiserum	Antibody dilution 100	150	200	300	400	600	800	1200	1600	2400	3200	4800	6400	12,800
16	I	A	250, 125, 62	0		0		0		0		0		0			
			31	+		±		0		0		0		0			
			16	+++		++		+		±		0		0			
			0	++++		+++		++		±		0		0			
18	I	A	125, 62	0		0		0		0		0		0			
			31	±		±		0		0		0		0			
			16	+		±		±		0		0		0			
			8	++		++		+		±		0		0			
			0	++++		+++		+++		+		0		0			
2	I	CBA	765		+++		+++		+++		++		±		0		
		A	765		0		0		0		0		0		0		
			0		+++		+++		+++		++		+		0		
4	I	CBA	500		+++		+++		++		++		+		0		
		A	500		0		0		0		0		0		0		
			0		++++		++		++		++		+		0		
10	I	C3H	500		++++		++++		+++		+		±		0		
		A	500		0		0		0		0		0		0		
			0		++++		++++		+++		++		+		0		
46	III	A	800			0		0		0		0		0		0	0
			400			+		+		±		0		0		0	0
			200			++		+		±		0		0		0	0
			100			+++		++		+		±		0		0	0
			0			+++++		+++++		++++		+++		++		+	±

1·0 ml. 1/50 antiserum, though exposure to less than 100 units diminished the titre appreciably.)

The specificity of the action of A-strain antigen was made equally apparent by using the " soluble " antigenic preparation (*i.e.* the antigenic extract immediately before its final centrifugation : see above) as an inhibitor of agglutination. Adjusted to a concentration of 100 units per ml., A-strain antigen reduced the titre by not less than 4 halving dilutions (Tables II, III), but an exactly parallel preparation from CBA or C3H lymphoid tissues was quite inactive (Table II).

TABLE II.—*Use of " Soluble " Antigenic Extracts from A-strain, CBA, and C3H Mice to Inhibit the Agglutination of A-strain Red Cells by Antiserum I*

Expt. No.	Antigen concentration in units/ml.*	Source of antigen	Antibody dilution 150	300	600	1200	2400
1	135	A	+	±	0	0	0
	0		+++	+++	++	++	+
2	77	A	++	+	±	0	0
	77	CBA	+++	+++	+++	++	+
	0		+++	+++	+++	++	+
4	100	A	+	±	0	0	0
	100	CBA	++++	+++	+++	++	+
	0		++++	++	++	++	+
10	100	A	+	±	0	0	0
	100	C3H	++++	++++	+++	+	±
	0		++++	++++	+++	++	+

* The final concentration in the 3-part system is one third of the stated figure.

We took advantage of the specific inhibitory power of this " soluble " preparation to find out what proportion of antigenic activity during the preparation of the final sediment remains in the supernatant fluid after the final centrifugation at 30,000 *g* for 60 min. The four readings summarized in Table III show that

TABLE III.—*Inhibition-titration of A-strain " Soluble " Antigen Before and After Centrifugation at* 30,000 *g for* 60 *min., Using Antiserum I*

Expt. No.	Antigen concentration in units/ml.	Pre- or post-centrifugation	75	150	300	600	1200	2400
13	100	pre		0	0	0	0	0
	100	post		+	+	±	0	0
	0			+++	+++	++	++	±
14	100	pre		0	0	0	0	0
	100	post		++++	+++	++	±	0
	0			+++++	+++	++	+	±
16*	100	pre	0	0	0	0	0	0
	50	pre	+	0	0	0	0	0
	25	pre	++	±	0	0	0	0
	12½	pre	+++	++	±	0	0	0
	100	post	+++	++	±	0	0	0
	0		+++++	+++	+	0	0	0
18*	100	pre	0	0	0	0	0	0
	50	pre	±	±	0	0	0	0
	25	pre	++	+++	++	+	0	0
	12½	pre	+++	++	++	+	±	0
	100	post	+++	+++	++	++	±	0
	0		+++	++++	+++	++	+	0

* The use of 1 per cent dextran is responsible for the low end-point titre in this experiment.

only about one-eighth of the activity remains in the supernatant fluid—a result which indicates that, at this stage of preparation, the power to absorb humoral antibody and to sensitize *in vivo* go hand in hand (Billingham *et al.*, 1958).

Direct effect of antiserum on the sensitizing power of antigen

The treatment of antigenic sediment with a great excess of specific antiserum *in vitro* had little if any effect upon its power to give rise to transplantation immunity *in vivo*. In each of 3 independent experiments, 1250 units of sedimented antigen were redispersed in 5·0 ml. freshly withdrawn and undiluted immune serum. After exposure for 30 min. the antigenic matter was recovered by centrifugation at 30,000 *g*, redispersed in Ringer-phosphate, and injected into 5 CBA mice at a dosage of 250 units per mouse. Five controls were treated in exactly the same way except for the replacement of antiserum by fresh normal serum from A-strain mice. All mice received test-grafts of A-strain skin 3 or 4 days after the injection of antigen, and the grafts were removed for histological examination 6 days later. The 2 sets of survival scores in the 3 experiments are recorded in Table IV : clearly there is no significant difference between them.

TABLE IV.—*Percentage of Epithelial Survival, at 6 days, in A-strain Skin Grafts on CBA Mice which had received 250 units of A-strain Antigen After its Exposure to Immune or to Normal Serum*

Expt No.	Titre of antiserum: Before absorption	Titre of antiserum: After absorption	Antigen treated with immune serum	Antigen treated with normal serum
403	1600	5120	25, 25, 5, 5, 5	50, 25, 25, 5, 5
405	1600	3200	100, 90, 75, 50, 5	50, 50, 25, 25, 5
415	6400	3200	50, 50, 5, 5, 5	50, 25, 25, 5, 5

These results are interesting because, as we shall record elsewhere—see Medawar (1959*b*) for a preliminary report—the injection of 1·0 ml. hyperimmune antiserum *in vivo* does indeed weaken the sensitivity produced by the injection of 250 units of antigen ; and the same is true when the injection of antigen precedes the injection of antiserum by as long as 3 days. Evidently this " enhancing" or " desensitizing " action of antiserum cannot be due to a simple direct inactivation of antigen by antibody. In the experiment referred to in Table IV the antigen : antibody absorption ratio was equivalent to only 5 units of antigen per 1·0 ml. 1/50 antiserum. It is not surprising, then, that the treatment did not lower the haemagglutinin titre of the antiserum ; on the contrary, the absorbed serum gave the higher titre in 2 experiments out of 3.

Production of antibodies by the injection of extracted antigens

Hildemann and Medawar (1959) were unable to satisfy themselves that the injection of isolated lymphoid nuclei or nuclear fragments could provoke the formation of humoral antibodies. Our present antigenic material can certainly do so. Five CBA mice received 250 units of A-strain antigen each by the intraperitoneal route on days 0, 5, 13, 18, 25 and 38, and were bled from the heart on the 45th day. Table V shows that haemagglutinins are formed in high titre. It also shows that the haemolytic titres fall far short of what would be expected of immunization by living lymphoid cells : the haemolytic titre of serum 2 (Table V)

TABLE V.—*Haemagglutinin and Haemolytic titres of the Sera of 5 CBA Mice which had received Six Injections of 250 units of Sedimented A-strain Antigen*

Serum No.	Agglutination endpoint	Haemolysis endpoint
1	2048	100
2	1024	<10
3	4096	200
4	2048	40
5	2048	50

was less than 10 where the agglutinin titre was about 1000, a fact which suggests that lysis and agglutination are not merely two different manifestations of the action of a single antibody.

A single intraperitoneal injection of 250 units of A-strain antigen into a CBA mouse is more than sufficient to bring about the total breakdown, within six days, of an A-strain skin graft transplanted 3 or 4 days later (Billingham *et al.*, 1958). Three of the 5 CBA mice hyperimmunized by extracted A-strain antigen were tested with A-strain skin 9 days after the last of their 6 injections of 250 units. Six days later the three grafts were found to carry some 50 per cent of surviving epithelium. The sensitivity of the recipients must therefore decline during the repeated administration of extracted antigen, just as it does during the repeated injection into CBA mice of living A-strain lymphoid cells (Medawar, 1959*b*). The very complex relationship between the survival time of a skin graft and the titre of humoral antibodies in its recipient will be discussed in another publication. For the present, it will be noticed that our results, so far as they go, do not uphold the tentative distinction between "T-antigens" and "H-antigens" in the terminology of Medawar (1959*a*) (see *Discussion*).

Stability of the antigenic sediment

Resistance to drying.—Drying in the frozen state does not abolish the absorptive power of the antigenic sediment. In spite of the fact that "lyophilized" tissues are regularly used to procure "enhancement"—*i.e.* to prolong the life of homografts by immunologically specific means—we now find that if the antigenic sediment is dried carefully its power to sensitize is not perceptibly impaired. In each of 5 independent trials, 1250 units of sedimented A-strain antigen were resuspended in 2–3 ml. water, shell-frozen at −79°, and dried for 3–4 hr. under vacuum (~0·001 mm. Hg) at a temperature not exceeding −5°. The material was reconstituted in Ringer-phosphate and injected into 5 CBA or C3H mice at a dosage of 250 units per mouse. Table VI summarizes the 6-day epithelial survival scores of A-strain skin grafts transplanted 3 days after the sensitizing injection. Their recipients were highly sensitive. In 5 further trials we found that drying could be continued overnight over phosphorus pentoxide under a vacuum of 0·01 mm. Hg without loss of sensitizing power, and that the dry preparation retained all or most of its activity after storage for more than 2 weeks under nitrogen at −20°. It follows that resistance to drying does not distinguish the power of an antigen to sensitize from its power to absorb humoral antibodies.

Effect of the periodate ion.—Kandutsch and Reinert-Wenck (1957) have shown that exposure to the periodate ion inactivates the antigens responsible for "enhancing" the growth of certain tumour homografts, and Billingham *et al.* (1958),

TABLE VI.—*Percentages of Surviving Epithelium, at 6 days, in A-strain Skin Homografts transplanted to CBA or C3H Mice Three Days after the injection of 250 units of Dried A-strain Antigenic Sediment*

(Expectation of survival of A-strain skin homografts 6 days after transplantation to normal CBA or C3H mice : 100.)

Expt. No.	"Lyophilized" antigen	Antigen further dried over P_2O_5	Notes
409	25, 5, 5, 0, 0		
411	25, 5, 5, 5, 0		
413	50, 0, 0, 0, 0		
416	50, 0, 0, 0, 0	0, 0, 0, 0, 0	
417	5, 0, 0, 0, 0	0, 0, 0, 0, 0	
420		0, 0, 0, 0	
423C		25, 25, 5, 0	Stored 7 days at 2°–3°.
423D		50, 5, 5, 5, 0	Stored 18 days at −20°.

found the same to be true of the sensitizing power of antigenic sediments. We have now re-investigated the matter, paying special attention to the possibility that products of the interaction between periodate and reducing agents might themselves have a damaging effect. Accordingly, 1250 units of A-strain antigen were exposed in the dark at 21° to 23° to sodium periodate in the presence of 0·1 M sodium phosphate pH 7·0 and 0·0375 M NaCl : the concentration of periodate was 0·005 M and its quantity (2 μM per 100 units of antigen). After 3 hr. 10 μM glycerol per 100 units of antigen were added to inactivate the periodate, and the antigenic matter was spun out at 30,000 *g* for 30 min. to eliminate the original reagents and their soluble reaction products. The sediment was resuspended in Ringer-phosphate and injected into CBA mice at a dosage of 250 units per mouse. A-strain skin grafts were transplanted 3 days after the injection of antigen and removed for examination 6 days later. Controls were of 2 kinds : those identical with the experimental series except that glycerol was added before instead of after the exposure to periodate, and those in which periodate was simply omitted from the reaction mixture. The results of 4 such experiments are summarized in Table VII, which reveals drastic impairment by active periodate but also some degree of impairment by inactivated periodate.

The power of periodate to weaken the absorptive power of sedimented antigen *in vitro* was investigated in much the same way. Antigenic sediment was suspended

TABLE VII.—*Percentages of Surviving Epithelium, at 6 days, in A-strain Skin Homografts Transplanted to CBA or C3H Mice 3 days after the injection of 250 units of Periodate-treated Antigen*

(See text for details of treatment and nature of controls.)

Expt. No.	Active periodate	Inactivated periodate	No periodate
406	100, 100, 75, 50, 25*	50, 5, 5, 5, 0	0, 0, 0, 0, 0
408	100, 100, 100, 50, 25*	75, 75, 50, 50, 50	50, 25, 25, 5, 0
412	100, 100, 75, 75, 75	50, 25, 5, 5, 5	0, 0, 0, 0, 0
414	100, 100, 100, 75, 50	75, 5, 5, 5, 0	0, 0, 0, 0, 0

* Poorly-healed grafts, probably underscored.

in 0·005 M sodium periodate (8 μM per 100 units of antigen) in the presence of 0·05 M sodium phosphate pH 7·0 and 0·075 M NaCl (0·0375 M in exp. 40). After 19 hr. in the refrigerator the periodate was inactivated by the addition of 40 μM glycerol per 100 units of antigen. Antigen was recovered by centrifugation as before, and then used to absorb antiserum II in the ratio 1·0 ml. 1/50 antiserum to 31 units of antigen (50 units in expt 41). In the control experiments, glycerol was added before the experiment began instead of after it was over. The results of three such sets of experiments, summarized in Table VIII, show that the titres of antibody exposed to a periodate-treated antigen are very little inferior to the titres of normal antibody.

TABLE VIII.—*Haemagglutinin Titres of Antiserum II after Absorption with Periodate Treated Antigen*

(For details of exposure and nature of controls, see text.)

Expt. No.	Nature of treatment	Antibody dilution 50	100	200	400	800	1600	3200
39	Active periodate	+++	+++	++	+	±	0	0
	Inactivated periodate	0	0	0	0	0	0	0
	No periodate	0	0	0	0	0	0	0
	Normal antibody	++++	++++	+++	+++	++	+	±
40	Active periodate		++++	+++	++	+	±	0
	Inactivated periodate		++	+	0	0	0	0
	Normal antibody		++++	++++	+++	++	+	±
41	Active periodate	++++	+++	+++	++	++	+	0
	Inactivated periodate	0	0	0	0	0	0	0
	Normal antibody	++++	++++	++++	+++	++	+	0

Effect of formaldehyde.—A suspension of 1250 units of A-strain antigen was exposed for 19 hr. at 2–3° to 4·0 ml. 0·1 M formaldehyde in 0·01 M sodium phosphate at pH 7·0. The antigen was recovered by centrifugation and injected into 5 C3H mice at a dosage of 250 units per mouse. This treatment had little if any effect on the sensitizing power of the antigen, for the 6-day epithelial survival scores of A-strain test grafts transplanted 3 days after the injection were, in 2 experiments, 5, 5, 5, 5, 0 and 25, 0, 0, 0, 0, respectively. The effect of such a treatment upon the power of A-strain antigen to absorb antiserum III is summarized in Table IX : something between 25 and 50 per cent of the original activity remained.

Effect of heat.—The exposure of a suspension of A-strain antigen in water to a temperature of 57° for 20 min. reduced its absorptive power to between 25 and 50 per cent of its original value. A number of not very systematic tests on antigenic preparations of various kinds showed that this degree of exposure to heat also weakened the power to sensitize *in vivo*, though without abolishing it completely. We did not pursue these experiments, because their interpretation is ambiguous : inactivation might be due to a very rapid enzymatic destruction in the temperature zone between 40°–50° during warming. On the other hand they led us to expect that heating to 100° for 4 min. would inactivate the antigen completely. So far as concerns its absorptive power, this expectation was fulfilled : Table X shows that antigen heated to 100° for 4 min. retained no trace whatsoever of its absorptive power. Most unexpectedly, however, 6 independent trials

TABLE IX.—*Haemagglutinating Activity of Antiserum III after Absorption with A-strain Antigen Exposed to M/10 Formaldehyde*

(For details of exposure, see text.)

Expt. No.	Treatment of antigen	Units of antigen per 1·0 ml. 1 : 50 antiserum	Antibody dilution 200	400	800	1600	3200	6400	12,800
45	Formaldehyde	400	+++	++	+	±	0	0	0
	Untreated	75	++++	++++	+++	++	+	±	0
	Unabsorbed	0	++++	++++	++++	+++	+++	+	0
46	Formaldehyde	400	++	+	+	0	0	0	0
	Untreated	800	0	0	0	0	0	0	0
	,,	400	+	+	±	0	0	0	0
	,,	200	++	+	±	0	0	0	0
	,,	100	+++	++	+	±	0	0	0
	Unabsorbed	0	+++++	+++++	++++	+++	++	+	±
50	Formaldehyde	500	++	+	+	0	0	0	0
	Untreated	500	+	0	0	0	0	0	0
	Unabsorbed	0	++++	++++	+++++	+++	++	+	±

TABLE X.—*Haemagglutinating Activity of Antisera absorbed with A-strain Antigen heated to 100° for 4 min.*

Expt. No.	Anti-serum	Treatment of antigen	Units antigen per 1·0 ml. 1 : 50 antiserum	Antibody dilution 150	300	600	1200	2400	
10	I	100°	500	++++	++++	+++	++	+	
		Nil	500	0	0	0	0	0	
		Unabs.	0	++++	++++	+++	++	+	
				200	400	800	1600	3200	6400
40	II	100°	125	++++	+++	+++	++	0	0
		Nil	31	++	0	0	0	0	0
		Unabs.	0	++++	+++	++	+	±	0
64	III	100°	1000	++++	+++	+++	++	++	+
		Unabs.	0	+++	++++	+++	++	++	+

have shown that antigen heated in aqueous suspension to 100° for 4 min. or even for 12 min. retains a small but clearly discernible fraction of its power to sensitize *in vivo*. These experiments are still in progress: the heated antigen might conceivably act through a general non-specific activation of its recipients' immunological response, a possibility that is now under investigation.

Effect of ethanol.—The effect of 75 per cent v/v aqueous ethanol upon A-strain antigen is to abolish its absorptive power or to reduce it to a level that cannot exceed one-eighth of its original value. This generalization applied with equal force to preparations of three kinds: (a) the precipitate formed almost instantly at 0° by the action of 75 per cent ethanol on a suspension of antigen in 0·05 M sodium acetate at pH 7·0; (b) the lipid-containing matter extracted into 75 per cent ethanol under the same conditions; and (c) an unfractionated preparation. Ethanol was removed from preparations of types (b) and (c) either on a rotary evaporator below room temperature or by dialysis against distilled water under

TABLE XI.—*Effect of 75 per cent v/v Ethanol on the Absorptive Power of A-strain Antigen*

Expt. No.	Anti-serum	Treatment of antigen	Units antigen per 1·0 ml. 1/50 antiserum	Antibody dilution 100	200	400	800	1600	3200	6400	12,800
21	I	Ethanol S	125	+++	++	+	0	0	0		
		Untreated	31	+	±	±	0	0	0		
		Unabsorbed	0	++++	+++	++	++	+	±		
22	II	Ethanol ER	300	++++	+++	+++	+++	++	0		
		Ethanol ED	500	++++	+++	+++	+++	0	0		
		Unabsorbed	0	++++	+++	+++	++	+	0		
39	II	Ethanol ER*	250	++	+++	±	0	0	0		
		Untreated	31	0	0	0	0	0	0		
		Unabsorbed	0	++++	+++	+++	++	+	±		
44	III	Ethanol TD	100	+++++	+++++	+++	+++	++	+	0	
		Untreated	31	++++	++++	+++	++	+	0	0	
		Unabsorbed	0	+++++	+++++	++++	+++	+++	+	±	
50	III	Ethanol TD	500		+++++	+++++	+++	+++	++		0
		Ethanol TR	500		+++++	+++++	+++	+++	++	++	±
		Untreated	500		+	0	0	0	0	0	0
		Unabsorbed	0		++++	++++	+++++	+++	++	+	±

S = Sediment precipitated by ethanol in the presence of acetate ions. E = Matter extracted into 75 per cent ethanol and not deposited by centrifugation at 5000 *g*. T = Unfractionated total preparation after exposure to ethanol. R = Ethanol removed by evaporation. D = Ethanol removed by dialysis.

* Extraction in aqueous ethanol: with no acetate to promote flocculation, the extract here contains matter that would have appeared in the sediment in Expt. 21.

nitrogen at a pressure of about 30 cm. Hg. The more informative experiments are summarized in Table XI.

Experiments too detailed to be summarized here show that preparations of type (a)—the precipitates formed instantly at 0° by adding 3 vols ethanol to 1 vol. antigen resuspended in 0·03 M to 0·05 M neutral sodium acetate or in 1·0 M $MgCl_2$—retained no more than a just perceptible fraction of their power to sensitize *in vivo*. (The mere exposure of a compact antigenic sediment to 75 per cent ethanol by diffusion at 2°–3° overnight was equally damaging.) Preparations of type (b), representing the matter extracted into 75 per cent ethanol, were quite inactive; and those "total" preparations of type (c) from which ethanol was removed by evaporation had no greater activity than preparations of type (a). However, preparations of type (c) from which ethanol had been removed by dialysis against distilled water overnight regained a high proportion of their sensitizing power—a property that distinguishes the absorptive from the sensitizing activities of the antigenic preparation, though the distinction may be trivial (see *Discussion*).

Inhibition Tests with Purified Substances

We have investigated the power of a number of oligo- and polysaccharides to inhibit the agglutination or lysis of A-strain red cells (*cf.* Albert and Lejeune-Ledant, 1959). Unhappily, most of the polysaccharides and some of the oligosaccharides promote agglutination non-specifically—a property presumably akin to that for which dextran itself was chosen as an adjuvant in the haemagglutination technique of Gorer and Mikulska (1954)—and other substances, like human blood-group A or B substance, are disqualified from the agglutination test because (see *Materials and Methods*) it makes use of human antibody-containing serum. Most of our tests are therefore of necessity based upon the inhibition of lysis.

Table XII is a list of the substances tested. With the following exceptions, all were inactive. A feeble inhibition of lysis (a reduction of the apparent titre to about one quarter, *i.e.* by 2 or 3 tubes) was given by 2 per cent solutions of 3 different samples of purified human blood-group A substance (though not by B, H, or Le^a substances); by Type XIV pneumococcal polysaccharide (though not by Types I, II, and V); and by a 2 per cent solution of a *Shigella shigae* polysaccharide. Rather stronger inhibition (with one sample, >4 and >5 tubes) was given by 2 different samples of purified hog gastric mucin with A but no H activity, whereas hog gastric mucins with H but very little A activity were quite ineffective. Thus the actively inhibitory substances were those distinguished by "Forssman" affinities. The degree of inhibition was unaffected by the addition of further complement. In view of the fact that human A-substance was active where such a close chemical relative as human B-substance was not, it seems possible, provisionally at least, to take these results at their face value. It may be added that the one sample we had of human M substance was also weakly inhibitory, but as human MN-substance was not we cannot at present build anything upon this observation.

DISCUSSION

The experiments described above show that, if the strains of donors and recipients are judiciously chosen, conventional serological methods can be used to study the physical and chemical properties of the iso-antigens associated with

TABLE XII.—*List of Purified or Chemically Defined Substances that have been Examined for their Power to Inhibit the Agglutination or Lysis of A-strain red Cells by Specific Antisera.*

A = Tested for agglutination-inhibition.
L = Tested for inhibition of lysis.

The final concentrations of the substances tested were one-third of the stated values in the agglutination tests and one-fourth of the stated values in the lytic tests.

A	D-Ribose 2 per cent.
A	2-Deoxy-D-ribose 2 per cent.
AL	D-Mannose 2 per cent.
AL	D-Galactose 2 per cent.
A	D-Glucose 2 per cent.
A	L-Fucose 2 per cent.
A	L-Rhamnose 2 per cent.
AL	α-Methyl-galactoside 2 per cent.
AL	β-Methyl-galactoside 2 per cent.
AL	α-Methyl-mannoside 2 per cent.
AL	Lactose 2 per cent.
AL	Raffinose 2 per cent.
AL	Fucosido-lactose 2 per cent.
L	Lacto-N-tetraose 1 per cent.
L	Lacto-difucotetraose 1 per cent.
L	Lacto-N-fucopentaose I 1 per cent.
L	Lacto-N-fucopentaose II 1 per cent.
L	Lacto-N-difucohexaose 1 per cent.
L	Lactosamine 1 per cent.
AL	N-Acetyl-D-glucosamine 2 per cent.
AL	N-Acetyl-D-galactosamine 2 per cent.
AL	Glucuronic acid, Na salt, 1 per cent.
AL	N-Glycolylneuraminic acid, Na salt, 1 per cent.
AL	N-Acetylneuraminic acid, Na salt, 1 per cent.
AL	O, N-Diacetylneuraminic acid, Na salt, 1 per cent.
L	Human Blood Group Substances A*, B, H, Le^a 2 per cent; M, N, MN 1 per cent.
L	Hog gastric mucin A-active*, H-active, 0·2–2 per cent.
L	Pneumococcal polysaccharides types I, II, V, XIV*, 1 per cent.
L	Bacterial polysaccharides *B. anthracis, B. megatherium, Past. pestis, Sh. shigae** 1–2 per cent.
L	Dextran 2 per cent.
L	Sialomucopolysaccharide 0·2 per cent.

* Substances with some activity: see text.

transplantation immunity in mice. Although strains A and CBA (or C3H) differ at many genetic loci controlling differences between the survival times of homografts (Barnes and Krohn, 1957), the activity of A-strain antigen *in vivo* and *in vitro* is dominated by "strong" antigenic differences at the Histocompatibility-2 locus (see Gorer, 1956, and Berrian and Jacobs, 1959: strain A has the composition H-2*a* and strains CBA and C3H the composition H-2*k*).

The absorption and inhibition tests bear directly on the properties of A-strain antigens that are associated with the formation of humoral antibodies. To what extent are they relevant to their sensitizing activity, *i.e.* to the fact that, after injection into mice of strains CBA or C3H, they curtail the life of A-strain skin grafts transplanted a few days later? All that can be said so far is that the two activities go hand in hand during the crude extractions so far attempted, and that, with two exceptions, physical or chemical treatments that reduce or abolish the

one activity will reduce or abolish the other. These results do not therefore uphold the distinction (Medawar, 1959) between " T-antigens " that sensitize and " H-antigens " that provoke the formation of humoral antibodies or inhibit their action *in vitro*. Drying and treatment by formaldehyde were equally innocuous and treatment by periodate or by ethanol were equally damaging to both activities. The two exceptions were these : (a) heating to 100° abolished the power to absorb antibody, but left a well-defined residue of sensitizing power ; and (b) it was possible to recover the sensitizing but not the absorptive activity by the prolonged dialysis of preparations exposed to 75 per cent ethanol. The differences, though genuine, may be unimportant : with such crude preparations, the effect of " fixation " by ethanol or heat may simply render combining sites inaccessible to antibody *in vitro* ; alternatively the heat-stable antigen may belong to a different antigenic system.

At present it is hardly possible to interpret the physical and chemical properties we have described in terms of chemical constitution. The clue offered by the inhibitory action of polysaccharides with Forssman affinities is a very tenuous one, but it points in the same direction as evidence from other sources (Kandutsch and Reinert-Wenck, 1957 ; Billingham *et al.*, 1958), viz : towards the complicity of carbohydrate substances in the immunologically specific reactions of the antigens associated with transplantation immunity in mice.

SUMMARY

Extracted cell-free antigens of A-strain origin that can sensitize CBA or C3H mice to homografts can also (a) excite the formation of humoral antibodies, and (b) specifically inhibit the agglutination or lysis of A-strain red cells by humoral antibodies *in vitro*. Their methods of preparation and their resistance to various physical and chemical treatments (drying, heating to 100°, exposure to periodate, ethanol or formaldehyde) do not justify drawing a distinction between sensitizing antigens and antigens that excite the formation of humoral antibodies. Polysaccharides with Forssman affinities have some discernible power to inhibit the lysis of A-strain red cells by specific antisera.

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SPLENOMEGALY AS A HOST RESPONSE IN GRAFT-VERSUS-HOST DISEASE

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THE graft-versus-host reaction is provoked by introducing homologous lymphoid cells into an immunologically defenceless recipient, for example by injecting an F1 hybrid between two inbred strains with cells from one of the parent strains. It was shown by Simonsen (1957) that spleen enlargement is an early and reliable sign of this reaction. Since the splenomegaly represents a cellular increase, rather than an increase of stromal elements or of fluid content, it has seemed natural to regard the added splenic mass as being largely composed of donor cells which have settled and proliferated there. Direct proof that this may occur has emerged from the studies in chicken embryos by Biggs and Payne (1959) using the sex chromosomes as markers. On the other hand, Davies and Doak (1960) injected mouse spleen cells labelled with a cytological marker (of mutational origin) into homologous newborn recipients and found that the mitoses observed in the spleen tissue during the ensuing period were almost entirely confined to cells of host type.

Whereas Davies and Doak estimated the proportion of donor cells among all dividing cells, we have attempted to estimate the proportion of donor cells among all immunologically competent cells, using the spleen assay method developed by Simonsen and Jensen (1959). At the same time the donor cells were characterized as to whether they were still capable of reacting against a secondary host of the same genotype as the primary host or whether they were only capable of reacting against the antigens of some third strain. Simonsen (1960) has claimed that adult donor cells can under certain conditions acquire tolerance of the primary host while otherwise retaining the power of immune response.

MATERIALS AND METHODS

Plan of the assays

A cell suspension is prepared from the supposedly chimerical spleen of an animal undergoing a graft-versus-host reaction, and injected into infant mice of such a type that they may react with splenomegaly to the donor component, but not to the host component. This object is achieved in the following two circumstances:

Serial passage.—The chimerical suspension is tested in secondary hosts isologous with the primary hosts. A positive reaction indicates activity of the donor component against host strain antigens.

Discriminant assay.—The suspension is tested in F1 hybrids between the original donor strain and some third strain. The hybrids are chosen to be immunologically mature enough to eliminate the host component of the suspension and thus to respond only to the donor component. A positive reaction indicates that the donor component has retained potential activity against antigens of the third strain.

The principles involved are illustrated in Fig. 1. An F1 hybrid between two inbred strains, A and B, suffers a graft-versus-host reaction from injected A cells. Its spleen enlarges, and contains its own hybrid cells (shown as crosses) with an admixture of donor cells (shown as circles). A suspension from the spleen is assayed both against isologous (AB) and semi-isologous (AC) infant hybrids. Provided that the latter are sufficiently mature immunologically to reject AB cells as foreign (in practice, after the 4th day of life) they will react with splenomegaly only to the presence of A cells in the mixture. In a more obvious fashion, the isologous AB hosts will react only to the A cells, to the extent that these donor cells have not acquired any degree of tolerance of B antigens during their sojourn in the primary host.

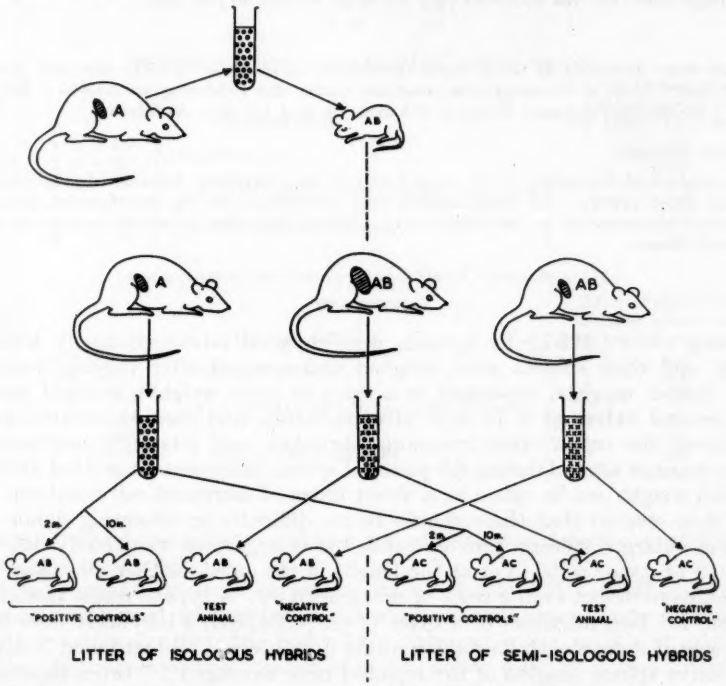


FIG. 1.—Diagrammatic representation of the discriminant spleen assay.

To control the assumptions stated above, litter-mates of the test animals must be injected with pure suspensions of AB cells. These serve as "negative controls" and provide the base-line against which to assess any splenomegaly observed in the test animals. "Positive controls" are also included. These consist of other litter-mates injected with pure suspensions of known numbers of A cells. If positive controls at more than one dose level are included, the degree of splenomegaly shown by the test animals can be interpreted in a quantitative fashion.

Mice

For the main series of assays, graft-versus-host disease was produced by injecting spleen cells from adult mice of the A inbred strain into F1 hybrids between A and C57BL. The semi-isologous hosts used in spleen assays were usually F1 hybrids between A and ASW, and sometimes between A and DBA/2. In a supplementary series C3H cells were injected

into (C3H × ST/A) F1 hybrids, and (C3H × DBA/2) F1 mice were used as semi-isologous hosts.

Cell suspensions

These were prepared by gentle trituration of the spleen in Hanks' solution (pH 7·4) with a glass piston blender, followed by passage through a fine-mesh stainless steel sieve. Cell counts were taken over a field of 1 sq. mm.; when the cells were very unevenly dispersed over the field, an additional three fields were counted and an average taken. The percentage of cells staining with 1/2000 Trypan Blue was frequently determined also, as an index of cell-damage, and this was found to vary between 10 and 30 per cent.

X-rays

These were delivered as whole-body irradiation at the rate of 150 r per min. given as a vertical beam from a Westinghouse machine under the following conditions: 230 kv. at 15 ma.; target-skin distance 50 cm.; 0·5 mm. Cu and 1·0 mm. Al filters.

Statistical analysis

The numerical treatment of the data followed the principles described by Michie, Woodruff and Zeiss (1961). All computation was performed on log-transformed data, which were finally reconverted to antilogarithms. Means therefore represent geometric and not arithmetic means.

RESULTS

Young (A × C57BL) F1 hybrids were injected intraperitoneally with adult A cells, and their spleens were weighed and assayed after varying intervals of time. Spleen weights, expressed as a ratio to body weights, reached levels 2–3 times control values at 8–10 days after injection, and then showed an irregular subsidence, the organ often becoming shrunken and relatively acellular. Cell density was not altered during the phase of splenic enlargement, so that an increase of spleen weight can be taken as a direct index of increased cell numbers.

It thus seemed that there should be no difficulty in detecting donor cells in assays of enlarged spleens, provided that the enlargement was chiefly due to proliferation of donor cells. Yet at the height of the splenomegaly our assay system was unable to detect even a trace of donor activity. A typical result is that shown in Table I. Two members of a litter of (A × C57BL) F1 hybrids were injected at the age of 8 days with 25 million adult A cells each. When killed 7 days later the relative spleen weights of the injected mice averaged 2·7 times those of their uninjected litter-mates. One of the enlarged spleens was used to provide a cell suspension for passage into young isologous and semi-isologous hybrids. No splenomegaly occurred in the secondary hosts. In the case of the semi-isologous hosts (A × ASW) we can utilize the positive control figures to estimate the admixture of immunologically competent donor cells in the inoculum as less than $\frac{2{\cdot}5}{32{\cdot}0}$, *i.e.* less than 7·8 per cent of all competent cells. In the case of the isologous hosts the corresponding upper bound is 9·4 per cent.

This result was quite typical of what was obtained from the whole series of some 50 assays of the spleens of recipient mice, done at intervals ranging from 24 hr. to 54 days after the initial injection of adult A spleen cells. The early period was the most thoroughly investigated, and in the case of the 24-hr. tests an attempt was made to force the concentration of donor cells above the threshold of detectability by the injection of totals of 3–4 × 10^7 cells divided between the intraperitoneal and intravenous routes, instead of the customary 5–25 × 10^6 i.p.

TABLE I.—*Specimen Discriminant Spleen Assay*

Primary hosts: (A × C57BL) F1 mice 8 days old.

Identity No.	Injected with		After 7-day interval: Body wt. (g.)	Spleen wt. (mg.)	
1	$2{\cdot}5 \times 10^7$	A cells	8·39	133	Presumed chimaera chosen for analysis
2	$2{\cdot}5 \times 10^7$	A cells	8·32	141	
3	—		8·00	50	Pooled to provide negative controls
4	—		8·14	52	Pooled to provide negative controls
5	—		8·26	53	Pooled to provide negative controls

Isologous test litter: (A × C57BL) F1 mice 7 days old.

Identity No.	Injected with	After 10-day interval, relative spleen weight (mg. spleen/10 g. body wt.)
1	$3{\cdot}2 \times 10^7$ Chimaera cells	56
2	$3{\cdot}2 \times 10^7$ Chimaera cells	54
3	$3{\cdot}2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	54
4	$3{\cdot}2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	51
5	1×10^7 A cells (positive controls)	161
6	3×10^6 A cells (positive controls)	80

Semi-isologous test litter: (A × ASW) 1 mice 5 days old.

Identity No.	Injected with	After 10-day interval, relative spleen weight (mg. spleen/10 g. body wt.)
1	$3{\cdot}2 \times 10^7$ Chimaera cells	65
2	$3{\cdot}2 \times 10^7$ Chimaera cells	64
3	$3{\cdot}2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	71
4	$3{\cdot}2 \times 10^7$ (A × C57BL) F1 cells (negative controls)	55
5	1×10^7 A cells (positive controls)	134
6	5×10^6 A cells (positive controls)	136
7	$2{\cdot}5 \times 10^6$ A cells (positive controls)	100

With one doubtful exception, no donor-cell activity was detected even following this intensive treatment.

The supplementary series of assays, in which the C3H→(C3H × ST/A) F1 combination was used, gave a rather different quantitative picture, although the qualitative conclusion, that the bulk of the enlarged spleen is of host origin, was the same. Estimates of the number of active donor cells, as a proportion of the total number of active cells present, are given in Table II. It appears not only

TABLE II.—*Proportion of C3H Donor Cells in 25 Chimaeric (C3H × ST/A) F1 Spleens, Estimated by Assay Against (C3H × DBA/2) F1 Mice*

Interval (days)	Estimated per cent anti-DBA activity	Average
1	0, 11, 12, 14, 21, 22, 36, 40	19·5
4	1, 10, 12, 15, 17, 39	15·7
11–28	0, 6, 9, 9, 13, 18	9·3
47–58	1, 4, 5, 7, 20	7·4

that these constitute a fairly small minority of the total, but also that they diminish in number relative to the host with the passage of time, even during the early period when the spleen is enlarging. Inocula ranging from 2×10^7 to

1×10^8 donor cells were used for establishing chimaerism, although 5×10^6 cells are sufficient in this combination to provoke a 2–3-fold enlargement of the spleen. The circumstances were thus more favourable for the detection of donor activity than in the main series described above.

Evidence from irradiated hosts

If, as suggested by the foregoing results, immunologically competent donor cells never constitute more than a small fraction of the spleen's competent cell population, even during the phase of enlargement, it seems likely that the greater part of the splenomegaly reaction is attributable to an increase of the host cell population. The alternative possibility is that donor cells have proliferated, but have become immunologically inert.

In order to decide this question we investigated the effect of X-irradiation of the host animal immediately before injection of spleen cells, with the object of depressing the postulated proliferation of host cells. Owing to the short survival time of infant mice subjected to higher dosages of irradiation, adult hybrid recipients were used for this purpose. In Table III the results of this experiment are

TABLE III.—*The Effects of X-irradiation and Injection of Parental-strain Spleen Cells on the Spleen Weight of (A × C57BL) F1 Hybrid Adults*

No. of spleen cells injected	Interval between injection and autopsy	Irradiated hosts			Non-irradiated hosts	
		X-ray dose	Injected with A spleen cells	Uninjected	Injected with A spleen cells	Uninjected
$8{\cdot}4 \times 10^7$	6 days	800 r	82 (5)*	47 (3)	84 (3)	40 (3)
9×10^7	7 days	600 r	91 (2)	40 (3)	116 (3)	
$10{\cdot}7 \times 10^7$	9 days	375 r	124 (3)	40 (3)	93 (4)	

* Spleen weights are expressed as mg./10 g. body weight. Figures (in parenthesis) indicate the number of mice in each group.

set out, from which it can be seen that when the dosage of donor cells is increased in proportion to body weight the splenomegaly phenomenon can be as easily elicited in adult as in infant mice. Surprisingly the table shows no effect of prior irradiation, even at 800 r, on the spleen weight in (A × C57BL) F1 hybrids. (This unexpected finding is contrary to our experience with both A strain mice and (C57BL × CBA) hybrids, in which a considerable reduction in spleen weight has invariably followed irradiation at this dosage.) However, spleen weight turned out to be no guide to the nucleated spleen cell population of irradiated (A × C57BL) F1 hybrid mice, for the spleens (even when enlarged following injection of A cells) were packed with erythrocytes which were evidently contributing substantially to the mass of the organ. Furthermore, an analysis of the total number of nucleated cells revealed that irradiation, especially at 800 r, had indeed been effective in depressing cellular proliferation following injection of A spleen cells. Relevant data are shown in Table IV.

Histologically, mice which had received 800 r alone showed a gross reduction in the number of nucleated cells present in the spleen with an accompanying increase of red cells (Fig. 2). The Malpighian follicles were small and contained foci of mature plasma cells (Fig. 3), whilst the network of reticulo-endothelial

TABLE IV.—*The Effects of X-irradiation and Injection of Parent-strain Spleen Cells on the Numbers of Nucleated Cells in the Spleens of (A × C57BL) F1 Hybrid Adults*

No. of spleen cells injected	Interval between injection and autopsy	Irradiated hosts				Non-irradiated hosts
		X-ray dose	Injected with A spleen cells	Uninjected	Injected—uninjected difference	Injected—uninjected difference
$8{\cdot}4 \times 10^7$	6 days	800 r	30*	18	12	55
9×10^7	6 days	600 r	43	15	28	76
$10{\cdot}7 \times 10^7$	9 days	375 r	87	34	53	78

* Cell numbers are expressed as millions per 10 g. body weight, and represent the means of batches of not less than three mice.

cells throughout the pulp was clearly revealed by the reduction in cell density. Similarly irradiated mice injected with A spleen cells possessed spleens which, although enlarged, also showed a sub-normal nucleated cell density and congestion with red cells. In contrast with uninjected irradiated mice, however, numerous pyroninophilic cells (some large and of blast type) were scattered more or less evenly throughout the red pulp (Fig. 4). A few of these cells were observed to be in mitosis, but far fewer than in the proliferating spleen cell population of an unirradiated mouse undergoing graft-versus-host reaction.

In view of this evidence of a considerable host contribution towards the increase in spleen cell population during graft-versus-host reaction, host irradiation was used to increase the sensitivity of spleen assays in detecting donor cell activity. Infant (A × C57BL) F1 hybrids, in which no donor activity was previously detectable, were irradiated to 600–800 r and injected with A spleen cells. The results of spleen analyses performed 5 days later (Table V) show clear-cut evidence of donor cell activity in all cases, with no obvious difference between the isologous and semi-isologous tests.

TABLE V.—*Evidence of Donor Cell Activity in the Spleen 5 Days After the Injection of A Spleen Cells into Irradiated Infant (A × C57BL) F1 Mice*

Chimaera No.	X-ray dose	Anti-C57BL		Anti-ASW	
		Activity of donor component	Estimated equivalent donor cell (per cent)	Activity of donor component	Estimated equivalent donor cell (per cent)
219/1–5*	800 r	Highly significant	<30	Significant	>36
220/1–2 and 221/1–3	600 r	Highly significant	Approx. 50	Not significant	<53
235/1–6 and 236/1–4	600 r	Highly significant	>27	Significant	<27

* Numbers after oblique stroke indicate pooled litter-mate chimaeras.

DISCUSSION

The conclusion of Davies and Doak (1960) that cellular proliferation in the spleen during graft-versus-host reaction in mice is largely of host origin has been confirmed by the present investigation, in which different strain combinations

and another experimental approach have been used. Our cell counts and histological observations on the effect of prior irradiation of the recipient on the cell population of the spleen supports this interpretation. So does the finding that immunologically competent donor cells only cross the threshold of detectability in one of our assay systems (A → A × C57BL) when the recipient has been irradiated, and that in the other (C3H→ C3H × ST/A) they constituted on average only 14 per cent of the total number of competent cells present. A similar proportion of donor cells was found by Michie, Woodruff and Zeiss (1961) in non-irradiated A-strain mice which had been injected neonatally with CBA spleen cells.

Little can be said at this stage about either the nature or the significance of the host response. Clearly it is unlikely to be immunological, since there are no antigens present in the system which are foreign to the host. Gorer and Boyse (1959) interpreted the cellular proliferation following injection of A-strain spleen cells into (C57BL × A) F1 hybrids as a histiocytic response. On the other hand, using the C57BL → (C57BL × CBA) F1 system, Howard (1961) found a predominance of pyroninophilic cells in the spleen which were morphologically indistinguishable from plasma cell precursors. Furthermore, neither these nor the increased number of reticulum cells were demonstrably phagocytic *in vivo* or argyrophilic in tissue sections.

Death from irradiation set an upper limit of 5 days to the time interval during which we could seek evidence of donor cell activity in infant irradiated recipients in the A → A × C57BL system, but in the non-irradiated hosts of the C3H → C3H × ST/A series donor cells were found to persist for a much longer time. It is noteworthy that after 5 days' sojourn within irradiated (A × C57BL) F1 mice, A cells showed no evidence of tolerance of C57BL antigens—in fact they were as active against hybrids containing C57BL as they were against hybrids containing third-party ASW antigens. This contrasts strongly with the other strain combination used, C3H → (C3H × ST/A) F1, for Simonsen (1961) found that after only 24 hr. within (C3H × ST/A) F1 mice, donor cells had become tolerant of the host antigens. Presumably the discrepancy is attributable to differences in the donor-host combinations employed.

SUMMARY

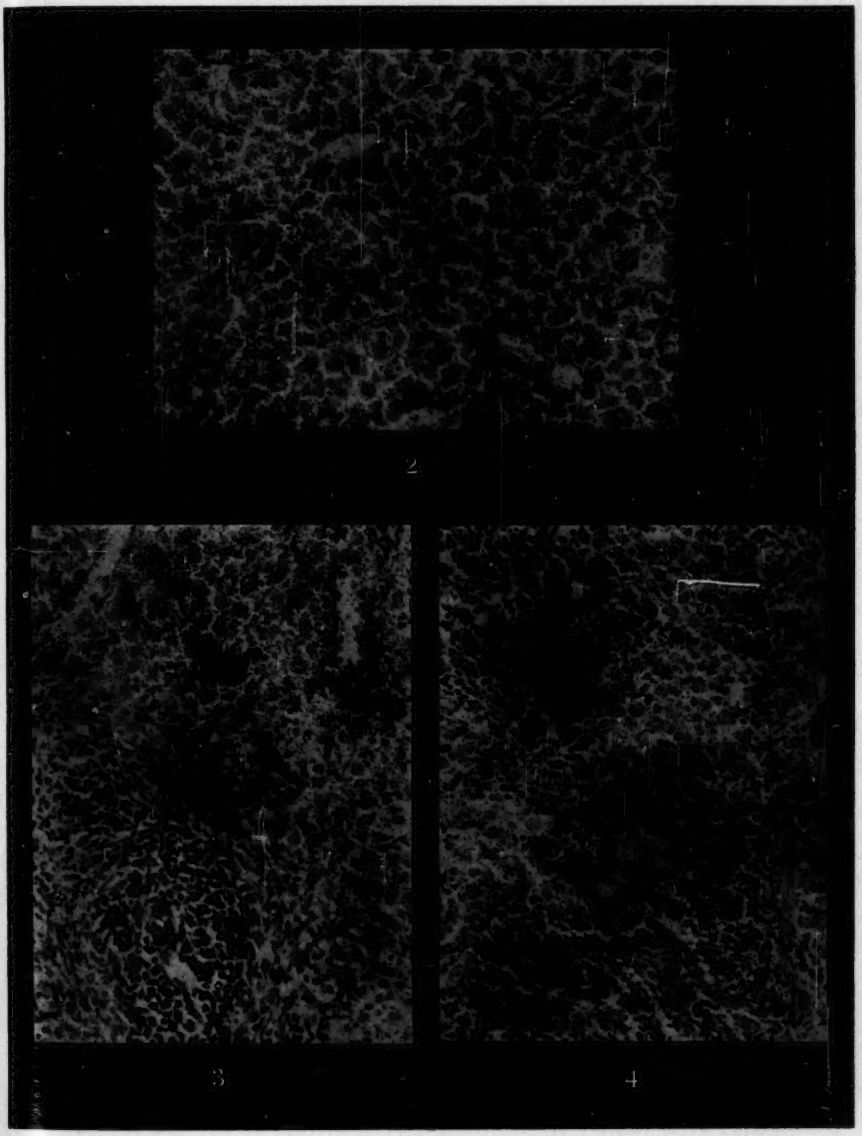
Graft-versus-host reaction was initiated in infant F1 hybrid mice by the injection of adult parental strain spleen cells. During the ensuing phase of spleen enlargement, the proportion of donor cells among all immunologically competent cells was estimated by means of the discriminant spleen assay of Simonsen and

EXPLANATION OF PLATE.

FIG. 2.—Red pulp of spleen of (A × C57BL) F1 mouse 6 days after whole-body irradiation (800 r). Note gross reduction in nucleated cell population and packed red cells. Pyronin-methyl green. ×177.

FIG. 3.—Malpighian follicle in spleen of mouse shown in Fig. 2. Note reduction in size, also focus of darkly staining mature plasma cells. Pyronin–methyl green. ×177.

FIG. 4.—Red pulp of spleen of (A × C57BL) F1 mouse 6 days after whole-body irradiation (800 r) followed by intravenous injection of $8{\cdot}4 \times 10^7$ A-strain spleen cells. Note pyroninophilic cells scattered throughout pulp (cf. Fig. 2). Pyronin–methyl green. ×177.



Howard, Michie and Simonsen.

Jensen (1959). Using the system A → (A × C57BL) F1, no donor cell activity could be detected during the period 1–54 days after injection of parental strain spleen cells. When the recipient had been previously irradiated (600–800 r), donor cells were readily detected at 5 days. Donor cells were detectable in the system C3H → (C3H × ST/A) F1 during the period 1–58 days after injection of C3H cells, without previous irradiation of the recipient, but they only constituted 14 per cent, on average, of immunological cells in the spleen.

The effect of previous irradiation of the recipient on the spleen enlargement which develops following injection of A spleen cells into adult (A × C57BL) F1 hybrids was examined histologically and by cell counting. Prior irradiation of the host was highly effective in depressing cellular proliferation in the spleen.

It is concluded that splenomegaly during graft-versus-host disease, at least in the two systems studied, is attributable in large degree to proliferation of host cells.

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THE PHAGOCYTOSIS OF UNIFORM POLYSTYRENE LATEX PARTICLES (PLP) BY THE RETICULO-ENDOTHELIAL SYSTEM (RES) IN THE RABBIT*

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THERE has been an increased interest in the phagocytic properties of the reticulo-endothelial system (RES), and the significance of phagocytosis by the RES in host defense mechanisms. There are several recent symposia in which the structure and function of the RES has been extensively considered (Halpern, Benacerraf and Delafresnaye, 1957; Heller, 1960*a*, 1960*b*). The recent studies on the RES have resulted in a better understanding of the conditions that govern the removal of a variety of colloidal particles from the circulation in different animals. As a consequence of these studies considerable new knowledge concerning the distribution of particles within organs and cells has been obtained.

Essentially phagocytosis by RE cells is governed by 2 broad considerations; the physiological condition of the host and the cells of the RES, and the physical and chemical characteristics of the particle that is presented to the RES. Although technically there are many ways to measure phagocytosis by the RES, all the procedures are variants of 2 methods; measuring the clearance of colloidal particles from the circulation, and location of the colloid in the various organs and within cells.

Despite the many investigations of phagocytosis by the RES, little information is available on the clearance from the circulation and on the organ and cellular distribution of suspensions of metabolically inert colloidal particles of known size and shape.

This report deals with the phagocytosis of uniform polystyrene latex particles (PLP). The study is divided into 2 phases; the clearance of PLP from the circulation as a function of particle size, dose and repeated challenge, and the organ and intracellular distribution of PLP.

MATERIALS AND METHODS

Albino rabbits of both sexes that weighed 2·0–3·5 kg. were used in these experiments. They were maintained on a standard diet with free access to water.

Polystyrene latex particles (PLP with diameters of 0·514, 0·557 and 1·171 μ) were obtained from the Dow Chemical Company (Midland, Michigan, U.S.A). The samples had a standard deviation by electron microscopic examination as shown in the Table.

Prior to use, the samples were washed several times in 0·15 M NaCl by centrifugation. After washing, stock suspensions were made in 0·15 M NaCl and the concentration determined

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TABLE

Particle size diameter	Standard deviation	Number of PLP per kg. bodyweight	Number of challenges given
0·514 μ	0·011 μ	$4{\cdot}6 \times 10^7$	1
		$1{\cdot}6 \times 10^{12}$	1
0·557 μ	0·0108 μ	$5{\cdot}5 \times 10^6$	1, 2 and 4
		$1{\cdot}09 \times 10^7$	4
		$2{\cdot}02 \times 10^7$	4
1·171 μ	0·014 μ	$0{\cdot}61 \times 10^7$	1
		$0{\cdot}88 \times 10^7$	1
		$1{\cdot}29 \times 10^7$	1

in a phase counting chamber. Dilutions were made in 0·15 M NaCl and counted as before. The samples did not contain foreign material by electron microscopic examination. There was no evidence of clumping.

The amount of PLP given to each animal was determined on the basis of body weight and is expressed in terms of the number of PLP per kg. The variation in concentration of PLP in any dose was ± 10 per cent. All injections were made into the marginal vein of the ear in animals that were not anaesthetized. The number of PLP that remained in the circulation as a function of time after injection was determined on blood withdrawn from the marginal vein of the other ear at periodic intervals using a phase contrast chamber.

The experiments were designed so that both clearance and the organ and intracellular distribution of PLP could be studied in the same animal.

Since PLP are soluble in the solvents ordinarily employed in the preparation of paraffin sections and in methacrylate monomers, other methods were adopted to study the distribution of PLP.

To obtain tissues for electron microscopy and for examination of thin sections by light microscopy the animals were anaesthetized with sodium pentobarbitol and the liver, spleen, mesenteric lymph nodes, lungs, and kidneys biopsied. The tissues were fixed for 1 hr. at 4° in 1 per cent osmium tetroxide buffered at pH 7·4 with veronal that contained sucrose (Caulfield, 1957). The tissues were gradually infiltrated and polymerized with Aquon (Epon Resin 812, Shell Chemical Co., New York City, New York, U.S.A.) according to the procedure of Gibbons (1959, 1960). Additional biopsy samples were infiltrated and embedded in methacrylate using one part methyl methacrylate and 9 parts butyl methacrylate with 1·5 per cent dibenzoyl peroxide as the catalyst.

The tissues embedded in Aquon and methacrylate were cut with a glass knife on a Porter Blum Microtome. Sections approximately 1000 Å thick were used for light microscopy and were stained with crystal violet and basic fuchsin (Moore, Mumaw and Schoenberg, 1960). Sections of 300–500 Å were used for electron microscopy.

By light microscopy the PLP were round refractile particles and were ovoid in the electron microscope. The distortion in the electron micrographs is very likely accounted for by the fixation, embedding and sectioning procedures. Particle size determinations from the optical specimens agreed closely with those obtained in the electron microscope.

Samples of the above organs and of the pancreas, adrenals, heart, skeletal muscle, skin, thymus, gastro-intestinal tract and bone marrow were immediately frozen in dry ice and acetone and stored at −40° for sectioning in the cryostat. These sections were stained with aqueous solutions of haematoxylin and eosin and mounted in cedar oil. In addition, tissues from all of the above organs were fixed in 10 per cent formalin, embedded in paraffin, and stained with haematoxylin and eosin for histological examination.

RESULTS

Clearance of PLP from the circulation

FIG. 1 illustrates the effect of concentration on the removal of PLP of 0·557 μ in diameter from the circulation after a single injection. There was an exponential clearance of PLP from the blood stream for all concentrations. Initially there was

a rapid removal of PLP. The majority were cleared within 60–90 min. After this time the removal of PLP from the circulation was slow. The concentration of PLP remaining in the circulation as the assymptote is approached was dependent on the dose. The higher the initial concentration given, the greater the number of particles remaining in the circulation up to 6 hr. In this interval there was almost a linear relationship between the concentration of PLP injected and that found in the circulation. In no case was it possible to demonstrate complete clearance. When the concentration of the PLP in the circulation was followed for

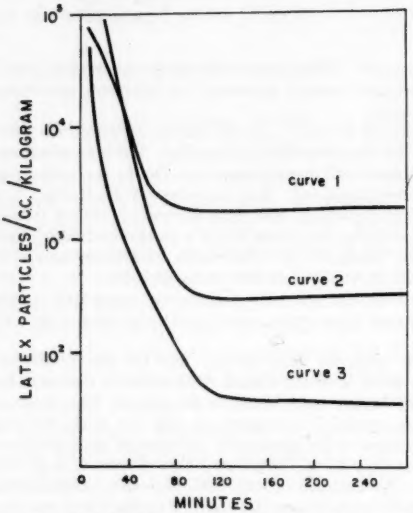

FIG. 1.—Logarithmic removal of PLP of 0·557 μ from the circulation as a function of time for several concentrations of latex.

Initial dose—Curve 1—$2{\cdot}02 \times 10^7$ PLP/kg.
Curve 2—$1{\cdot}09 \times 10^7$ PLP/kg.
Curve 3—$5{\cdot}5 \times 10^6$ PLP/kg.

long periods (up to 18 days) after injection, the same level of circulating PLP (300–400 PLP/ml./kg.) was found in all the rabbits independent of dose.

The particles of 0·514 and 1·171 μ in diameter did not show any significant differences in clearance from the 0·557 μ particles for a single dose. An assymptotic level was reached at approximately the same time for all the particles at comparable concentrations.

The effects of additional challenges of the same dose and size of PLP at various time intervals on the clearance are shown in Figs. 2, 3 and 4. Although the clearance curves for subsequent challenges follow the same general pattern as those for a single dose several differences were noted. At the lower concentrations of PLP ($5{\cdot}5 \times 10^6$ PLP/kg.—Fig. 2) the clearance curve for the second challenge on the 11th day closely followed that of the first challenge. Initially there was a rapid fall in circulating PLP with both curves approaching a common assymptote. Additional challenges on the 27th and 49th days showed a rapid initial phase of clearance that

levelled off at a higher concentration of PLP in the circulation at the end of 90 min. (4×10^2 PLP/ml. versus 5×10^3 PLP/ml.) and remained close to that level.

Subsequent challenges in the same animals for the higher doses ($1{\cdot}09 \times 10^7$ and $2{\cdot}02 \times 10^7$ PLP/kg.) showed a rapid initial decay phase that reached a common but higher assymptote than the first dose (Figs. 3 and 4).

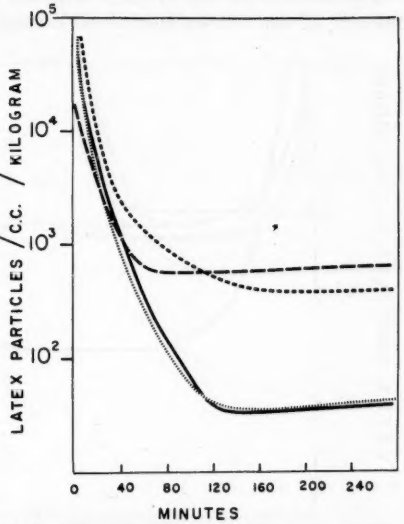

FIG. 2.—The effect of multiple challenges of 0·557 μ PLP of the same concentration ($5{\cdot}5 \times 10^6$ PLP/kg.) on the clearance from the circulation.

Initial challenge.
Challenge 49 days later.
Challenge 27 days later.
Challenge 11 days later.

The organ and cellular distribution of PLP

Polystyrene latex particles could not be identified as such in the paraffin and methacrylate embedded tissue because of their solubility in the solvents used to process the tissues. There were small spherical spaces in the RE cells where the PLP had been. When the frozen tissue was sectioned in the cryostat and stained with aqueous solutions of haematoxylin and eosin, the PLP were preserved and could be seen with the light microscope. The particles could be identified and localized best in tissues that were processed in Aquon.

PLP were found with varying frequency in the cytoplasm of RE cells and endothelial cells and within vascular spaces in the spleen, liver, lungs, kidneys and lymph nodes. They were not found in the adrenals, pancreas, bone marrow, skeletal muscle, thymus, heart, digestive tract and skin. There was no evidence of hyperplasia of the RES.

In the previous section it was shown that a two-fold difference in particle size, and a several fold variation in concentration did not materially alter the clearance

of PLP from the circulation. Their distribution in the tissues and the intracellular location was not modified by changes of concentration or multiple doses.

Tissues from animals that received the different sized particles and which were killed shortly after an injection showed differences in the distribution of PLP in the various organs. In animals that were killed at longer periods after the injection the distribution was the same for the different sized particles.

Light microscopic examination of the Aquon sections showed that a few of the cells lining the sinusoids of the spleen contained numerous PLP, Fig. 5. The

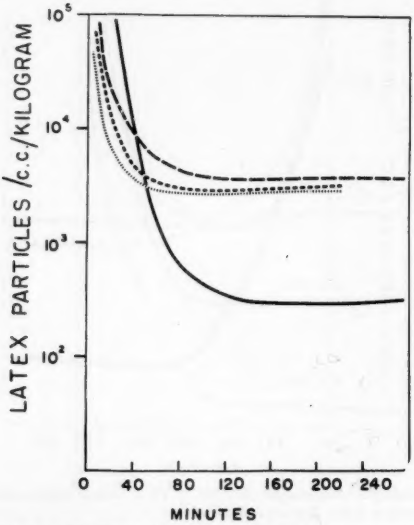

FIG. 3.—The effect of multiple challenges of 0·557 μ PLP of the same concentration ($1{\cdot}09 \times 10^7$ PLP/kg.) on the clearance from the circulation.

Initial challenge.
Challenge 57 days later.
Challenge 32 days later.
Challenge 16 days later.

number in the individual cells lining the sinusoids and within the lumen of the sinusoids was greater with the larger number of doses of PLP injected. In addition there was an increase of particles in the spleen as a function of time of examination after injection. Frequently there were several hundred per cell per section. There was no evidence of abnormal breakdown or proliferation of the RE cells. It should be pointed out that even with large doses only a small percentage of the cells contained PLP.

The lymph nodes in only one animal had a few macrophages with PLP in their cytoplasm.

Latex particles were found in some of the Kupffer cells ; many did not contain PLP, Fig. 6. There were fewer particles in the cytoplasm of the Kupffer cells, that contained latex, compared to those found in the cytoplasm of the cells lining the sinusoids of the spleen. The number of Kupffer cells that contained PLP increased as a function of increasing dose. However, even at large doses only a

small fraction of the cells contained latex. Those animals that were examined at longer times after injection showed fewer Kupffer cells with PLP. This was paralleled by the increase in the number of PLP found in the RE cells of the spleen and the larger numbers of cells in the spleen that contained PLP. Rarely was a particle found free in the sinusoids of the liver.

In the lungs PLP were difficult to find in the cytoplasm of the cells. Shortly after an injection, the majority of the particles found in the lung were in the lumen of the capillaries of the alveolar walls, particularly when the smaller particles

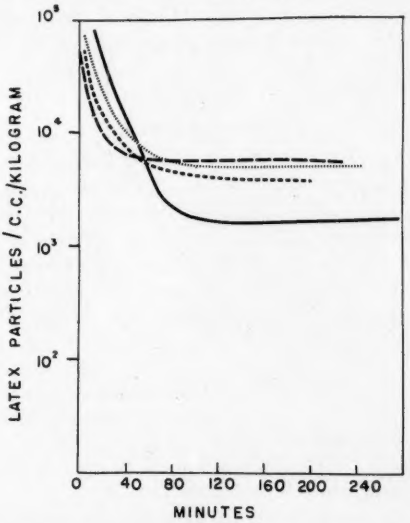

FIG. 4.—The effect of multiple challenges of 0·557 μ PLP of the same concentration ($2{\cdot}02 \times 10^7$ PLP/kg.) on the clearance from the circulation.

Initial challenge.
Challenge 52 days later.
Challenge 32 days later.
Challenge 16 days later.

(0·514 and 0·557 μ) were injected. With time the capillary spaces contained very few particles.

The initial accumulation of PLP in the lumen of the capillaries of the lungs was not due to the formation of emboli. There was no evidence of clumping of particles nor was there evidence of tissue alterations that would ordinarily accompany emboli.

The kidneys from animals that were killed within an hour after a single injection of PLP had a few particles within the lumen of the capillary loops of a few of the glomeruli. Rarely did endothelial cells contain PLP. The particles could not be identified in the kidneys of animals that were killed several hours after receiving the PLP or in those animals that had received multiple doses.

Examination of the cells of the peripheral circulation from the rabbits that received PLP intravenously, showed that polymorphonuclear leucocytes and

monocytes phagocytosed all of the sizes of PLP used. Only an occasional cell from the peripheral blood contained PLP. The number of particles per cell varied considerably. In studies using the coverslip procedure it was also demonstrated that polymorphonuclear leucocytes and monocytes actively phagocytosed PLP.

Electron microscopic observations confirmed the general distribution of PLP. The PLP within cells were confined to the cytoplasm (Fig. 7). The particles were distinct and in some instances there was a suggestion that they were enclosed or coated with some material (Fig. 8). The RE cells of the spleen contained the largest number of PLP on a per cell per section basis. The cells frequently had several hundred particles per cell/per section. This was followed numerically by the Kupffer cells of the liver. There was an insignificant number of PLP in the cells of the other organs.

DISCUSSION

In the past few years much has been learned about the phagocytic activity of the cells of the RES from studies of the clearance of many kinds of colloidal particles from the circulation. Despite the extensive investigations surprisingly little information is available on the effects of particle size, dose and repeated challenge on the blood clearance and the organ and cellular distribution of homogeneous suspensions of uniform colloidal particles of known size. To study the effect of particle size, dose and repeated challenge on the blood clearance and the cellular distribution of colloids in the cells of the RES, polystyrene latex particles were used.

Certain conditions must be met whether phagocytic function is measured by the removal of particles from the circulation or measured by the accumulation of these particles in the cells that comprise the RES. Ideally the particles should be uniform in size (Gofman, 1949 ; Benacerraf, Biozzi, Halpern and Stiffel, 1957 ; Dobson, 1957 ; Neukomm, Lerch and Jallut, 1957) and not lost from the circulation other than by phagocytosis. In addition the particles should be metabolically inert and not toxic to the cells.

The PLP used in these experiments have the following characteristics : 1. they are of uniform size and shape, 2. there is no evidence that they are lost from the circulation except by phagocytosis, 3. the particles so far as is known are metabolically inert and not toxic to the host, 4. there is no evidence that they cause a histological alteration in any of the organs in which they are deposited, and 5. they

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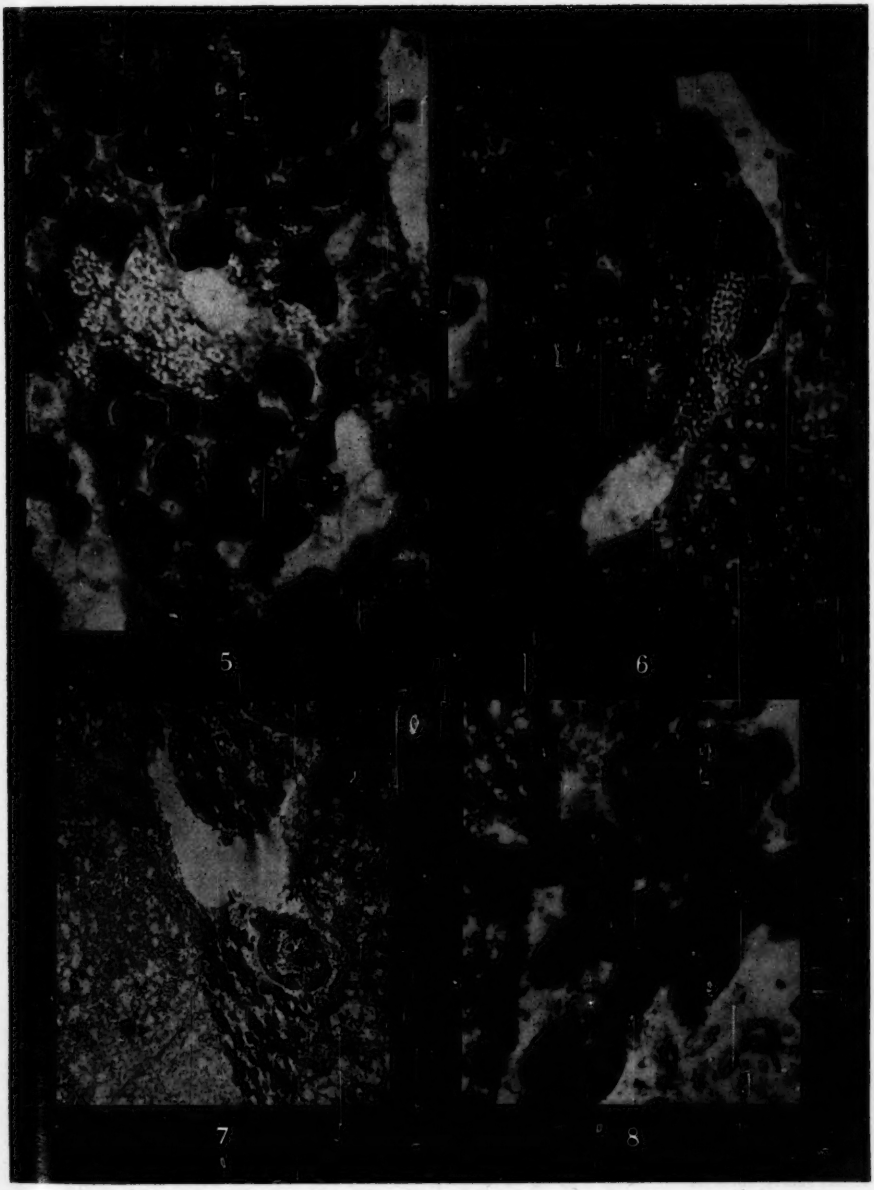
EXPLANATION OF PLATE

FIG. 5.—Thin section from the spleen embedded in Aquon from a rabbit that received four doses of 0·557 μ particles of latex. The latex appears as round, refractile particles. The clearance curves for this rabbit are shown in Fig. 3. A few of the RE cells contain particles. Crystal violet and basic fuchsin $\times 1320$.

FIG. 6.—Thin section from the liver embedded in Aquon from the same rabbit as Fig. 5. Three Kupffer cells are shown, only one of which contains numerous particles of latex in the cytoplasm. Crystal violet and basic fuchsin $\times 1320$.

FIG. 7.—An electron micrograph of liver embedded in Aquon from the same rabbit, Fig. 5. Two Kupffer cells with numerous particles of latex (oval, electron dense particles) in their cytoplasm. $\times 2230$.

FIG. 8.—An electron micrograph of the same liver as Fig. 7, which illustrates several discreet particles of latex. Some of these are enclosed or coated by some material. $\times 22{,}690$.



Schoenberg, Gilman, Mumaw and Moore.

are readily identified in the circulation and within cells. In view of these characteristics the PLP offer a model system to study the phagocytic phenomenon.

When PLP of the same shape but of different sizes are injected in comparable numbers per kg. of body weight the clearance from the circulation is very nearly the same. Within the limits of these experiments particle size is not the determinant in clearance. This is contrary to the results of some, who have found differences in clearance dependent upon particle size (Gofman, 1949; Dobson, Gofman, Jones, Kelly and Walker, 1949; Sheppard, Jordan and Hahn, 1951; Zilversmit, Boyd and Brucer, 1952; Dobson, 1957; Neukomm, Lerch and Jallut, 1957). These experiments are usually based upon clearance of colloids that have a relatively broad size distribution and where the shape of the particles is generally not well known. It should also be pointed out that most of the data have been obtained with colloidal particles of smaller size than those used in these studies.

The semi-logarithmic plot of the concentration of PLP versus time always showed a rapid, nearly linear initial clearance phase followed by an extremely slow rate of clearance—" tail effect ". Particles were always present in the circulation and persisted for a long time at a constant level. These results were independent of the size of the particles and amount of PLP presented to the animals. The particles that were found in the circulation after long times were microscopically the same as the particles injected. A slowly disappearing portion of clearance curves have been found for a variety of colloids. Benacerraf *et al.*, (1957) using coagulated albumin globulin aggregates tagged with varying amounts of ^{131}I, have suggested that there is a difference in the " homogeneity of phagocytosis " as a function of the degree of iodination. Since it has been shown that these colloids have different antigenic properties it is not fair to assume that they are directly comparable. Dobson (1957) has treated the "tail effect" largely on the basis of a non-homogeneous distribution of particle size and suggested that the tail portion of the colloidal clearance curve is due to the inefficient phagocytosis of small particles. The results reported by Dobson (1957) are consistent with the probable broad distribution of particle sizes that were used. It should be emphasized that the PLP used in the present study were homogeneous within the limits of electron microscopic measurement.

When multiple equal doses of the same concentration of the same sized particles are used the subsequent clearance curves are superimposable independent of the number of particles injected with no change in the initial phase of clearance. Nor was there any change in the tail portion of the curves. This suggests that a steady state for clearance has been reached and also shows that there was no inhibitory effect on phagocytosis from prior injections of PLP into the same animal.

Within the dose ranges used in these experiments there was no indication that there was a more efficient clearance of small doses. This is different from clearance studies that show a more efficient clearance of small doses and an inefficient clearance of large doses for other colloids (Dobson and Jones, 1952; Dobson, 1957 Benacerraf and Miescher, 1960). This variation in clearance has sometimes been explained by a functional alteration of the RE cells produced by the injected material.

The PLP are not toxic; there is no indication that they are metabolized; additional injections are always cleared to a base level; and, in all instances there are numerous RE cells that do not contain PLP. The persistence of the " tail effect " cannot be explained on overloading or injury of the RE cells. In addition,

the particles are uniform in size, shape and composition, so that variation in size cannot account for the " tail effect ", nor is it likely that there is a selective phagocytosis of any of these particles.

The small circulating level of latex reached and maintained in these experiments may be explained by a constant renewal of the RE cells that have participated in phagocytosis. There is a regular and constant renewal of most cells in the body. This is usually not taken into consideration in studies on the clearance of particles from the circulation by the RE cells. If the colloid is not metabolized or excreted and the number of cells of the RES or their physiological state are not changed, the injected material should never be completely cleared from the blood, if there is a reasonably rapid turnover of these cells. In fact, clearance of particles may be used as a rough measure of half life of cells.

This turnover of cells can also explain the shift of PLP from one organ to another, *e.g.* liver to spleen. The progressive drop in the number of Kupffer cells containing PLP with time would follow the turnover of that population of cells.

With the number of PLP administered in these experiments, no evidence was found that the Kupffer cells were saturated with PLP before phagocytosis occurred in the spleen nor was there evidence that the large particles were preferentially removed by the liver. The number of particles within the cells and the number of cells that contained PLP was always greater in the spleen than in the liver. The tendency of particles to be localized in the liver when small numbers are given may be due to the disproportion of blood distributed between liver and spleen in the course of a complete circulation.

Perhaps the most significant finding and at the same time the most perplexing problem introduced by these experiments is that only a small number of the cells lining the sinusoids of the spleen and of the Kupffer cells of the liver contain PLP. These cells are always considered as components of the RES and consequently it is presumed that the vast majority of these cells behave as phagocytes. In these experiments only a limited number of these cells have PLP in their cytoplasm. RE cells immediately adjacent to those RE cells that contain PLP have not phagocytosed the particles. The current concepts of phagocytosis would have led one to expect all or most of the cells to contain variable quantities of PLP, especially in view of the number of PLP given to the animals, the number in some of the cells, and the constant exposure of all the cells to PLP in the circulation.

Only a portion of the RE cells are participating in phagocytosis. This suggests that blocking of all the functional capacities of the RES is unlikely, if not impossible, with colloidal particles, and that within the limits of these experiments the principal function of RE cells may vary at any time. The functional definition of the RES based on phagocytosis must be re-examined. It is no longer possible to define the RES solely on the basis of phagocytic function.

SUMMARY

The phagocytosis of particles of polystyrene latex of several uniform sizes was studied in albino rabbits. The clearance from the circulation and the organ and cellular distribution of the particles were investigated.

The size of the particles did not alter the clearance curves, and complete clearance of particles from the circulation never occurred during the course of these experiments. Multiple challenges in the same animal did not block the phagocytic

function of the RES, and there was no indication that the number of particles injected altered the efficiency of clearance.

The RE cells of the liver and spleen contained the majority of particles. The ultimate distribution of latex was not influenced by the size of the particles or the number of particles injected.

Only a small percentage of the RE cells of the liver and spleen contained particles of latex regardless of the number given to the animals.

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THE CYTOTOXIC FACTOR IN HASHIMOTO'S DISEASE AND ITS INCIDENCE IN OTHER THYROID DISEASES

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In a previous communication (Pulvertaft, Doniach, Roitt and Hudson, 1959) we demonstrated that the serum of patients with Hashimoto's disease is cytotoxic to monolayer cultures of human thyroid cells. These observations have now been confirmed (Irvine, 1960; Goudie and McCallum, unpublished; Forbes, Roitt, Doniach and Solomon, unpublished), and we report further studies on the characteristics of this phenomenon and on the incidence of the cytotoxic factor in patients with Hashimoto's disease, thyrotoxicosis, non-toxic colloid goitre and thyroid cancer.

MATERIAL AND METHODS

Patients

These were drawn from a surgical clinic at the Middlesex Hospital and the diagnosis was established in each case by clinical examination supported by ^{131}I uptake studies, basal metabolic rate and serum cholesterol estimations. All patients were tested for thyroid antibodies. The tanned cell test (TRC) and precipitin reaction in agar were used for detecting anti-thyroglobulin, while the complement fixation test using thyrotoxic thyroid extract was employed to detect anti-microsomal antibodies (Roitt and Doniach, 1958). The CFT was done on serum dilutions from 1/4–1/512 and was controlled for organ-specificity by parallel tests done with human liver extracts. Coons' fluorescent antibody technique was used for the detection of antibodies to the second antigen of the thyroid colloid CA2, (Balfour, Doniach, Roitt and Couchman, 1961).

Storage of sera

After separation the sera were stored at −20° and in general they were used within 2 months. It is now clear that the cytotoxic factor is stable for many months and is active in tissue cultures provided sufficient complement is present.

Tissue culture technique

As much thyroid tissue as can be obtained from the operating theatre is immersed in a balanced salt solution (*e.g.* Hanks' fluid) and forwarded to the laboratory as soon as possible. A delay of 2-3 hr. before processing does no harm, but tissue maintained at 4° overnight has given a poor yield of viable cells. As much blood as possible is washed off, and the tissue is then finely minced with crossed scalpels. Mechanical mincers have always given a poor yield. The minced tissue is washed three times in balanced salt solution and then placed in the enzyme solution to disperse the cells. No advantage is gained by any form of mechanical agitation; the mince is simply immersed in the fluid in a screw-capped container and shaken by hand at 15 min. intervals.

Of the many enzymes available, a mixture of 0·25 per cent trypsin and 0·1 per cent collagenase gave the best yield of cells. About 2 g. of mince is added to 20 ml. of enzyme mixture, but the proportions are not critical. The mixture is placed in a water bath at 37°

for at least 1 hr. and samples are examined at intervals under the microscope to control the degree of dispersal. The tissue does not break down to single cells, but to disc-shaped aggregates which probably represent the glandular acini. The material is then filtered through muslin to remove the mucinous mass which remains, and centrifuged at 300 r.p.m. for 2 min. Nearly all the debris and blood cells remain in the supernatant fluid which is carefully removed. The deposit of epithelial cells is re-suspended in 10 ml. of salt mixture and centrifuged again for 2 min. This deposit is then suspended in Parker's 199 (Morgan, Morton and Parker, 1950) adding a drop at a time, until the required concentration of cells is reached. It is not possible to give exact guidance; 3–5 cell aggregates per field should be seen when a drop is observed under a 10× objective. Although a wide latitude is permissible, no cytotoxic effect will be found if an excess of cells is used. This can easily be shown by doing tests using doubling concentrations of cells; beyond a certain concentration full survival of cells is found even with strongly cytotoxic sera.

The patients' sera are used fresh, or stored at −20°. Fresh human serum is collected on the day of test as a source of complement, which is undoubtedly necessary. Guinea-pig complement has on occasion been found to be toxic, and in order to maintain continuity the same individual's complement has been used throughout. The serum contained 10 MHD/ml. of complement (Wyler, 1929). It is essential to use known positive and negative sera as controls in the cytotoxic test. In order to avoid doing a large series of tests uselessly if the selected thyroid cells should prove insensitive, a preliminary test may be done using a known positive serum, and watching effects on a warm stage. With experience toxic changes can soon be noticed. The required number of tubes or bottles are set up; to each is added 0·4 ml. of thyroid cells (mixing thoroughly with each pipette full, as the cells quickly settle), 0·2 ml. of fresh normal human serum and 0·2 ml. of test serum. The mixture, in our method, is then placed in a slide culture chamber, made by fixing a plastic ring to the slide with high vacuum silicone grease, and sealing with a cover-slip (Pulvertaft, 1959). When incubated cover-slip down with normal serum, the cells begin to form a monolayer after 18 hr. at 37° and are firmly fixed to the glass. Cytoplasmic spread and adhesion indeed begin in 1 hr. No tissue has been found which adheres or spreads so quickly as does the thyroid. These slide chambers give excellent optical results, but so long as containers are sealed their shape is immaterial, although good clear glass is essential.

RESULTS

Cytotoxic effect

When examined after 18 hr. positive sera are found to have entirely prevented cellular adhesion, and such cells as are visible are mere ghosts.

In order to establish the existence of a cytotoxic effect, no more need be done. However, the effect of cytotoxic sera on cells grown in normal sera can be studied by substituting a mixture of 25 per cent positive serum, 25 per cent fresh normal serum and 50 per cent salt medium. The effect is practically instantaneous in many cases, and always manifest within 1 hr. at 37°. The first effect is on filamentous mitochondria, which in the thyroid are very long though scanty, and poorly contrasted by phase. They become much darker and shrink to a fraction of their former size. They then become dark spheres, which quickly swell and become translucent. At this stage the cytoplasmic projections retract, and the cell becomes spherical. The nucleus of the thyroid has normally a faint degree of darkness; it becomes entirely translucent, and the nuclear membrane becomes sharper in outline. This change is noted, by phase, in all dying cells.

The cell aggregates now become detached from the glass, and eventually sink. If, as is often the case, the preparation contains polymorphs and monocytes, these move towards the dying cells as soon as the positive serum is added. It is clear that positive chemotaxis develops instantaneously; polymorphs and monocytes as opposed to lymphocytes, which will be discussed separately, are not attracted to thyroid cells growing in normal sera.

Positive sera damage only thyroid cells. Polymorphs and fibroblasts are unaffected. Eosinophils have never been seen in a thyroid preparation.

In the primary culture test positive sera kill all the cells added, up to a maximum as stated above. However, when cells grown in normal sera are subsequently exposed to toxic sera, only a proportion, usually those on the periphery of aggregates, are killed. Moreover, after 24 hr. growth in normal sera cells lose their sensitivity to toxic sera, and when treated again with trypsin, they do not regain it; this might be because an antigen is lost in culture, or because normal serum adds a protective agent.

Selection of thyroid cells for test

First experiments were done with cells from Hashimoto thyroids but these are rarely available, and the yield of epithelial cells is too poor to carry out many tests. It is clear that not all pathological thyroids are affected by positive sera, though all can readily be grown *in vitro*. Colloid goitre cells are in general unsatisfactory and the majority grow well in the most potent sera. As a rule cells from the fleshy glands of patients with Graves' disease are suitable; excellent results were also obtained from a case of foetal adenoma.

One case, diagnosed as carcinoma of thyroid clinically, and "small cell carcinoma" on histological grounds, showed when dispersed by trypsin no epithelial cells, but only small motile single cells, mostly monocytes and lymphocytes. Neither the patient's own serum nor known positive sera had any effect on these cells. This method of examination is valuable in classifying this obscure neoplasm, which on tissue culture grounds belongs clearly to the reticuloses.

In one case cells from a secondary deposit of carcinoma of thyroid in a lymph node gave positive results with the patient's own serum and with known positive cytotoxic sera, while surviving well in normal controls. In this connection it should be recorded that on 2 occasions, cells from secondary deposits of malignant melanoma failed to survive in the patient's own serum while growing excellently in normal serum; and on submitting the surviving cells to the patient's serum, although there was no immediate effect, in 24 hr. all the melanoma cells were found to be dead. Malignant melanoma is the easiest neoplasm to grow *in vitro*, and the observations appear to indicate the presence of a cytotoxic agent in the serum. However, such results are exceptional, and in a large number of cases of human malignancy, including leukaemia of all kinds, there was no evidence of a cytotoxic agent. Since nitrogen mustard and similar drugs are often used today in treatment, it is of course, important to exclude their use; in one case, a neuroblastoma, a positive result was traced to this source.

It is obvious that the experimental methods here described might well be applied in other diseases which are thought to have an auto-immune basis since the dispersed cells from synovial membrane might be tested in rheumatic conditions and dispersed renal cells in nephritis. At present only preliminary tests have been performed.

The behaviour of lymphocytes

In the histological diagnosis of Hashimoto's disease the large number of lymphocytes is a characteristic feature. In fact lymphocytes are nearly always present in tissue culture of a pathological thyroid but sometimes in such numbers

as to form a thick deposit after centrifuging the dispersed cells. When the thyroid cells spread on the cover-slip the lymphocytes move between them and the glass, which is strange, since the thyroid cells are firmly attached. They survive as long as the thyroid cells, at least 6 weeks, and never divide.

In Hashimoto's disease they are firmly adherent to clumps of cells and move along pseudopodia. They circulate vigorously in between the cells in the aggregates, and it is not possible to be certain that they are not actually inside cells. This phenomenon has been described as emperipolesis (Humble, Jayne and Pulvertaft, 1956). In the case of cultures of embryonic tissue and of intestinal cells, lymphocytes are certainly inside other cells (Trowell, 1958).

This adhesion of lymphocytes has recently been investigated experimentally by Sharp and Burwell (1960), using homografts in the rabbit's ear. They describe the adhesion of lymphocytes to monocytes in material from the lymph node draining the ear, and allude to it as peripolesis. It seems that such associations are found in the process of immunisation, and we have observed it in inflamed lymph node cultures. We have noted in cinema films that when lymphocytes and other motile cells, including amoebae, leave the surface of cells to which they have become attached, a fine filament of cytoplasm connects them for several 100 μ; a similar filament is found in the motile form of toxoplasma. It is obvious therefore that when cells depart from intimate contact the cytoplasm of one becomes sequestrated onto the other, and if the cytoplasm contains antibody, this may be one way in which it is conveyed. Antibody is also present, of course, free in the serum. In tissue culture sequestration of cytoplasm by all cells, including lymphocytes, is of common occurrence, and may be a source of free serum antibody.

Effect of complement

When Hashimoto's sera are heated to 56° for 30 min., cytotoxic activity is lost. It has now been found that addition of sufficient fresh human or guinea-pig serum restores activity to the heated sera suggesting that complement is implicated. Further, when serum was decomplemented by absorption with a washed specific precipitate of human γ-globulin and its rabbit antibody, cytotoxic activity was abolished without heating, but was regained on addition of fresh normal serum.

When the cultures are set up in 50 per cent serum concentration, the complement level in the test serum is usually adequate provided the sera are not more than 2–3 months old. However it is preferable to use the pathological serum at a concentration of 25 per cent and to add an equal volume of a fresh normal serum known to support growth and to contain at least 10 M.H.D. of complement per ml.

Some properties of the cytotoxic factor

The activity of pathological sera was undiminished after dialysis for 24 hr. against Hanks' fluid or against normal human serum while no cytotoxic effect could be shown in the external dialysing fluid in both instances.

Absorption of a Hashimoto serum with an excess of purified human thyroglobulin failed to inhibit the cytotoxic activity and this parallels the finding that a proportion of cytotoxic sera are negative in the sensitive tanned red cell test for thyroglobulin antibodies. Conversely some sera with low or moderate TRC titres did not affect the growth of thyroid cultures. All sera giving an organ-

specific complement fixation test with thyrotoxic thyroid extracts were cytotoxic but activity was also observed in many sera negative by complement fixation.

The cytotoxic factor is specific to thyroid epithelial cells. Not only are the polymorphonuclears, monocytes, lymphocytes and fibroblasts present in the thyroid cultures unaffected by pathological sera, but cultures of human stomach, kidney and synovial membranes grow well in Hashimoto serum. Cells derived from sheep, pig or monkey thyroid gland were insensitive to the cytotoxic factor.

Enzyme dispersal

It is not possible to obtain appropriate cell suspensions without the use of enzymes. Thyroid tissue, however finely minced by mechanical mincers or cutters, has never yielded a suspension which gave clear cut results.

Collagenase, discussed for cell dispersal by Rinaldini (1959), has not been widely used in tissue culture or in immunology, but is of the greatest value. (Rinaldini found it less effective than trypsin, probably because of the preparation used—we use collagenase purchased from the Worthington Biochemical Corporation, New Jersey, U.S.A.) Collagenase has also been recommended by Lasfargues (1957). Few adult tissues can be dispersed with trypsin and its use in the culture of human neoplasms is very limited. Collagenase, however, makes it possible to disperse such tissues as adult human uterine muscle, synovial membrane, human sarcomata and carcinomata, adrenals, testes and salivary glands with most satisfactory results.

The only disadvantage is that it is easily contaminated, by moulds, bacteria and in one case, even by amoeba, in spite of the addition of penicillin and streptomycin.

Collagenase and trypsin have quite separate properties. For example, thyroid cells adherent to glass are quickly removed by trypsin while collagenase has no effect whatsoever. Conversely, trypsin has no power to dislodge cells embedded in collagen, *e.g.* carcinoma of breast, while collagenase is most effective (Ebner, Hoover, Hagemen and Larson, 1961). The best yield of cells from all tissues, including the thyroid, comes from their combined use. In Hashimoto's disease there is much collagen in the thyroid and with trypsin alone the yield of cells is very poor. In this case the addition of collagenase is important.

Further work requires to be done on the function of enzymes in the cytotoxic phenomenon *in vitro*. Results to date have been equivocal but in one fully controlled test, thyroid cells dispersed with trypsin were sensitive to known positive sera, and when dispersed with collagenase were certainly insensitive. Papain and ficin appear to be similar to trypsin in their action, but optimal concentrations have not been worked out. Experiments are not altogether simple, as with all enzymes, cells die if digestion is unduly prolonged. Untrypsinized thyroid fragments grown on rafts of collagen were not affected by the presence of a potent Hashimoto serum in the medium.

Incidence of cytotoxic factor in thyroid diseases

The results of a clinical survey are summarised in the Table. The highest incidence was observed in Hashimoto patients whose serum always affected thyroid cells. The majority of these were strongly cytotoxic but 3 of the Hashimoto sera were weak and only inhibited growth in the most sensitive glands. Two weak sera

TABLE.—*Incidence of Cytotoxic Factor in Sera from Patients with Thyroid Disorders*

Diagnosis	Number of patients	Cytotoxic activity +	Cytotoxic activity −
Hashimoto's disease	50	47	3
Thyrotoxicosis	35	25	10
Non-toxic colloid goitre	38	6	32
Thyroid carcinoma	4	2	2
Controls : blood donors	30	1	29

were from patients treated with thyroid hormone for several years with complete regression of the goitre at the time of testing. In the third case the serum was from a euthyroid man with a moderately large lymphadenoid goitre and positive precipitin test. In a few cases of long-standing primary myxoedema, very weak or no cytotoxic effects could be obtained.

Twenty-five of 35 thyrotoxic patients (64 per cent) had cytotoxic factor and 13 of the cytotoxic sera gave negative results by CFT and TRC though they showed the presence of antibodies by the fluorescent technique. The presence of cytotoxic factor did not correlate with the subsequent development of post-operative myxoedema in the patients, and in one case with cytotoxic antibody, hyperthyroidism recurred after a course of carbimazole. Several patients had cytotoxic factor after thyroidectomy. Among patients with colloid nodular goitres, 6 : 38 were cytotoxic and histological examination showed focal thyroiditis lesions. Four cases of thyroid carcinoma were tested, of which 2 were cytotoxic, even to their own tumour.

Serum from cases of systemic lupus erythematosus, Sjogren's syndrome and patients with extensive burns did not influence the growth of thyroid cells.

Thirty blood donor sera were tested as controls and one was found to be cytotoxic though it did not react in the TRC or CF tests. Two other blood donors gave weak reactions in the TRC but did not kill thyroid cells. On a separate occasion a blood donor serum was cytotoxic and on investigation the patient was found to have a CFT titre of 1 : 256 and a strongly positive test for CA2 antibodies, though his thyroid was not enlarged. ^{131}I uptake tests showed a " low reserve " thyroid and the patient became overtly hypothyroid 1 yr. after the condition was discovered.

DISCUSSION

The antibody nature of the cytotoxic factor is indicated by its organ and species specificity and by its requirement for complement. Our previous failure to restore cytotoxicity to heated sera by addition of guinea-pig complement was misleading since further studies by ourselves and others (Irvine, 1960 ; Goudie and McCallum, unpublished) amply demonstrated the role of complement in this reaction. The earlier results may be attributed to adding insufficient amounts of complement to the tests. Earlier interpretations about the stability of the cytotoxic factor must now be revised. The factor itself is stable at 56° and persists for at least 2 yr. at −20°.

The high level of microsomal antigen (Roitt, Doniach, Wilson and Couchman, 1960) in thyrotoxic glands as compared with the normal thyroid and their greater susceptibility to cytotoxic factor would suggest that the same immune system is

involved in the tissue culture reaction. The fact that cytotoxic factor was found in the serum of patients who give negative results by CFT with microsomal antigen does not invalidate this assumption since Forbes *et al.* (unpublished) have now shown cytoplasmic thyroid antibodies in such cases by a more sensitive technique. If this conclusion is correct the sensitivity of cultures made from Hashimoto glands would imply that the few functioning epithelial cells in these goitres have a high level of " microsomal " antigen. Indeed, high content of microsomal antigen is not a prerogative of the thyrotoxic thyroid in view of the comparable levels found in dyshormonogenetic goitres. We have not yet had the opportunity of testing dyshormonogenetic glands by tissue culture and it will be interesting to see if they prove susceptible to the cytotoxic factor. The failure of the monkey thyroid to be affected in tissue culture in spite of the good cross-reaction of its antigens with the human microsomal antibody is not unexpected in view of the insensitivity of the euthyroid human gland.

The finding of cytotoxic factor in a high proportion of thyrotoxic patients parallels the incidence of thyroid antibodies and of focal lymphadenoid lesions in Graves' disease. In non-toxic colloid goitres, both cytotoxic factor and focal thyroiditis lesions are less common (Doniach, Roitt and Hudson, 1961). Since these focal lesions are rarely progressive, it would appear that the cytotoxic factor is not primarily responsible for the tissue destruction occurring in diffuse thyroiditis. The inability of Hashimoto sera to affect the survival of non-trypsinized fragments of thyroid gland in raft cultures is further evidence that the cytotoxic factor does not injure normal cells.

Trypsin has been shown to reduce the volume of the cell cytoplasm in monolayer cultures (Weiss, 1958) and it is possible that it uncovers certain antigenic sites not normally available at the surface of intact cells. Exposure of hidden antigenic groups is thought to be the mechanism by which agglutination reactions occur with trypsinized red cells in certain cases of haemolytic anaemia and rhesus incompatibility. The requirement for treatment with a proteolytic enzyme would suggest that the cytotoxic factor could only be pathogenic *in vivo* if the cell surface were previously injured in some way in auto-immune thyroiditis. How this initial injury is brought about is at present obscure. The interesting behaviour of the lymphocytes in cultures of Hashimoto goitres suggests that these cells may be sensitized and since lymphoid cells are thought to effect the tissue breakdown in experimental auto-allergic conditions and homograft rejection, it is conceivable that they damage the cell surface in Hashimoto's disease thereby potentiating the action of the cytotoxic antibody.

SUMMARY

Trypsinized human thyroid cells in monolayer cultures are killed by Hashimoto sera in the presence of complement. If complement is inactivated by heating or absorbed out by an indifferent antigen-antibody complex, the cytotoxic effect is abolished. The factor is species and organ specific and only consistently affects cells from thyrotoxic and Hashimoto goitres. The factor is strongest in Hashimoto sera but is also present in low concentration in two-thirds of thyrotoxic sera and to a lesser extent in other thyroid diseases.

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STUDIES WITH *VIBRIO CHOLERAE* IN THE LIGATED LOOP OF THE RABBIT INTESTINE

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THE ligated loop of the rabbit small intestine has been utilized as a model by De and Chatterjee (1953) and by Jenkin and Rowley (1959) to study certain aspects of the pathogenesis of cholera. The former workers found 24 hr. after the injection of *Vibrio cholerae* that the ligated segment was distended with a protein-rich fluid which contained numerous desquamated epithelial cells. They observed oedema, congestion, and necrosis of the summits of the villi, but stated that nowhere in the wall was there any evidence of inflammatory cellular infiltration. These authors believed that the protein-rich fluid had escaped from the necrosed portion of the mucosa and suggested that this also occurred in human disease.

Jenkin and Rowley (1959) considered 3 factors to be implicated in the pathogenesis of the experimental disease and proposed that they might play a role in human cholera; these factors were the production of lactic acid by the bacteria during growth, mucinase enzyme, and lipopolysaccharide endotoxin. Lactic acid production was considered to be the most important of these factors. The purpose of this communication is to record the results of experiments in which rabbits infected with *V. cholerae* were killed sequentially and studied in the early as well as the late stage of the experimental infection.

MATERIALS AND METHODS

Cultures; *V. cholerae* (Inaba) strain 20–A–67 was obtained from the type culture collection of the Walter Reed Army Institute of Research. The culture has been maintained in the lyophilized state. For challenge 0·25 ml. volumes of 16 hr. nutrient agar grown cultures diluted in brain heart infusion broth were employed. The challenge dose varied from 5×10^7 $-1{\cdot}5 \times 10^8$ cells.

Surgical procedure; Rabbits weighing 1·5–2 kg. were used in all experiments. The technique of preparing the intestinal segments was similar to that of Jenkin and Rowley (1959). The animals were anaesthetized with sodium pentobarbital and ether, and employing sterile technique the abdominal cavity was entered. Segments of ileum were prepared using the tip of the appendix as a reference point. Bacterial challenges were introduced into the segments by means of a syringe fitted with a 27 gauge needle. Animals were killed under ether anesthesia at frequent intervals from 4–24 hr. after infection. All of the intestinal fluid from infected loops and 10 ml. samples from the non-infected normal bowel above these loops were rapidly withdrawn. Tissue specimens of the gut wall of infected loops were secured from 116 rabbits for histological analysis. Adjacent segments of non-infected bowel served as controls. As an additional control the surgical procedure was performed on 6 animals but the loop was not infected. Four of these rabbits were killed and examined histologically 8 hr. and the remaining two 20 hr. after surgery. Fluid did not accumulate in these uninfected segments but was obtained from the region above the loops. All tissues were fixed in 10 per cent neutral

buffered formalin and processed in a routine manner which included the rolling up of the bowel over a forceps to make a spool of tissue.

Sodium lactate ; Sodium lactate for human injection manufactured by Baxter Laboratories, Morton Grove, Illinois, was used. This is prepared as a M/6 solution. Each ml. contained 18·6 mg. sodium lactate. Sodium lactate syrup prepared by the Fisher Scientific Co., Fairlawn, New Jersey, was also employed. This was 60 per cent by weight sodium lactate by our assay technique.

Chemical determinations : Assays for total protein and lactic acid were carried out by the technique described by Hawk, Oser and Summerson (1947).

Results

Four hours following infection fluid began to accumulate in the ligated loop, increasing rapidly and averaging approximately 20 ml. by 8 hr., 27 ml. by 12 hr., and 34 ml. by 20 hr. Focal haemorrhagic necrosis of the wall of the infected loop. infrequent until 10 hr., increased in severity and by 16 hr. had progressed in most animals to severe haemorrhagic necrosis of the entire wall of the segment.

At 4 hr. after infection, the earliest stage examined microscopically, a focal vascular reaction was observed which rapidly became diffuse. There was congestion of the capillary network of the villi and separation of tissue elements of the tunica propria by a protein-rich oedema fluid which also filled the lymphatics. There were no recognizable alterations in the villus epithelium or its basement membrane except for depletion of mucus from the goblet cells of crypts and villi.

In the course of the inflammatory reaction the villi tended to become blunted, thickened, shortened and irregular. Congestion of villus capillaries, deeper vessels and lymphatics was more marked at 8 hr. post infection. Segmented leucocytes in the tunica propria increased in numbers. By 12 hr. they frequently formed sheets in the deeper mucosa and submucosa ; leucocytes adhered to the endothelium and surrounded vessels in increasing numbers. At times these inflammatory cells filled crypt glands and spaces between villi. Pyknotic nuclear fragments, debris of necrotic cells and proteinaceous precipitate accumulated in the tunica propria near the tips of villi.

Starting at 8 hr. the epithelial cells especially toward villus tips changed from the usual high columnar to cuboidal shape or became even more flattened. They revealed increased pleomorphism, apparent narrowing of striated border, increased basophilia of cytoplasm and increased necrocytosis. The continuity of the epithelial lining was preserved in the initial phase of the acute enteritis.

Focal necrosis of the mucosa of infected loops was very infrequent during the first 10 hr. of the infection, but thereafter rapidly increased in frequency and extent, being present in nearly all animals examined 16 or more hr. after challenge. The necrosis was superimposed upon the above described pattern of non-specific acute inflammation. This necrosis began at the tips of villi as focal necrocytosis of epithelial cells and focal microscopic ulcerations which were covered by accumulation of fibrin and segmented leucocytes. In more severe cases the necrosis involved either the complete thickness of the mucosa or the entire intestinal wall and was frequently accompanied by haemorrhage and in some cases by thrombosis of vessels. The marked leucocytic infiltrate which was present in the early stages of the infection was absent in a few animals examined late in the course of the disease or after death from infection. Phagocytosing monocytes were prominent in the tunica propria of some animals examined at a late stage of their infection.

Massive desquamation of epithelium was only observed as a post mortem autolytic change.

A histologically distinct lesion showing a "sprue-like" pattern occurred in 8 animals. In these animals the mucosal architecture was greatly altered. Villi were markedly shortened and thickened and tended to fuse with one another. The crypt glands were greatly increased in depth. In some areas this produced flattening of the surface and complete obliteration of the villous pattern. The surface epithelium though intact, revealed degenerative changes similar to those described above. Crypt glands occasionally were obstructed, dilated and filled with leucocytes and cellular debris. The lamina propria frequently contained an excess of large mononuclear cells. In addition, there was congestion, cellular infiltrate and plasma exudation.

Table I lists the condition of the ligated loops of intestine at various time intervals after inoculation with regard to presence of acute inflammation and severity of necrosis of the intestinal wall.

The fluid from the infected segments of 36 representative animals was collected for chemical analysis. All segments contained large amounts of fluid. The animals were classified into 3 groups depending upon the histological alteration of the segment: acute inflammation only, acute inflammation with focal necrosis, and inflammation with marked necrosis. In addition, intestinal fluid was analyzed from 2 of the 6 sham-operated but uninfected rabbits referred to earlier. Also fluid from the small intestines was harvested from several normal rabbits which had no previous surgical procedure.

The results of the lactic acid determinations are summarized in Table II.

EXPLANATION OF PLATES

All sections were stained with haematoxylin and eosin.

FIG. 1.—Intestinal villus, rabbit ileum, non-infected loop. Note empty central lacteal, collapsed capillary bed and sparsity of cells in the tunica propria. The epithelial covering is uniform and regular. There is a slight degree of transmigration of lymphocytes. ×384.

FIG. 2.—Intestinal villi, rabbit ileum, cholera-infected loop, 3½ hr. after challenge. Note inflammatory oedema of the tunica, filling of the lacteal with protein-rich fluid and cellular debris. There is slight hyperaemia and inflammatory cell infiltrate. Transmigration of inflammatory cells has increased. ×384.

FIG. 3.—Intestinal villus, rabbit ileum, cholera-infected loop, 8 hr. after challenge. Note hyperaemia of capillary bed, inflammatory oedema and increased accumulation of inflammatory cells. The continuity of the epithelial lining is preserved, however there are beginning degenerative changes of the epithelial cells. ×451.

FIG. 4.—Cholera-infected loop, rabbit ileum, 8 hr. after challenge, border of mucosa and submucosa. Note acute inflammatory reaction with marked oedema and hyperaemia. Segmented leucocytes show evidence of margination, are in the process of migrating from the vessels into the surrounding tissues. ×451.

FIG. 5.—Intestinal villus, cholera-infected loop, rabbit ileum, 12 hr. after challenge. Note exudative and proliferative inflammatory reaction and plugging of subepithelial capillary by segmented leucocytes. The epithelium shows more advanced degenerative changes. ×451

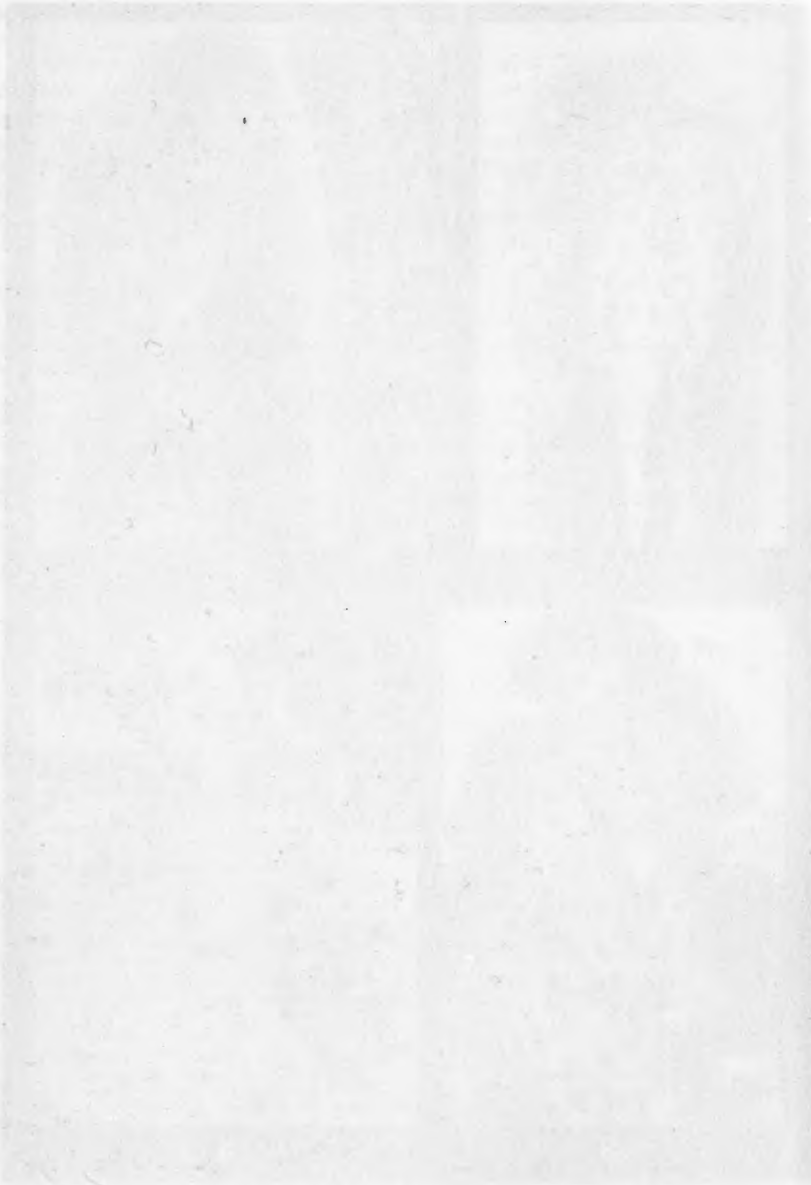
FIG. 6.—Intestinal wall, cholera-infected loop, rabbit ileum, 12 hr. after challenge. Note severe hyperaemia, leucocytic infiltrate and focal haemorrhagic necrosis of villi. ×112.

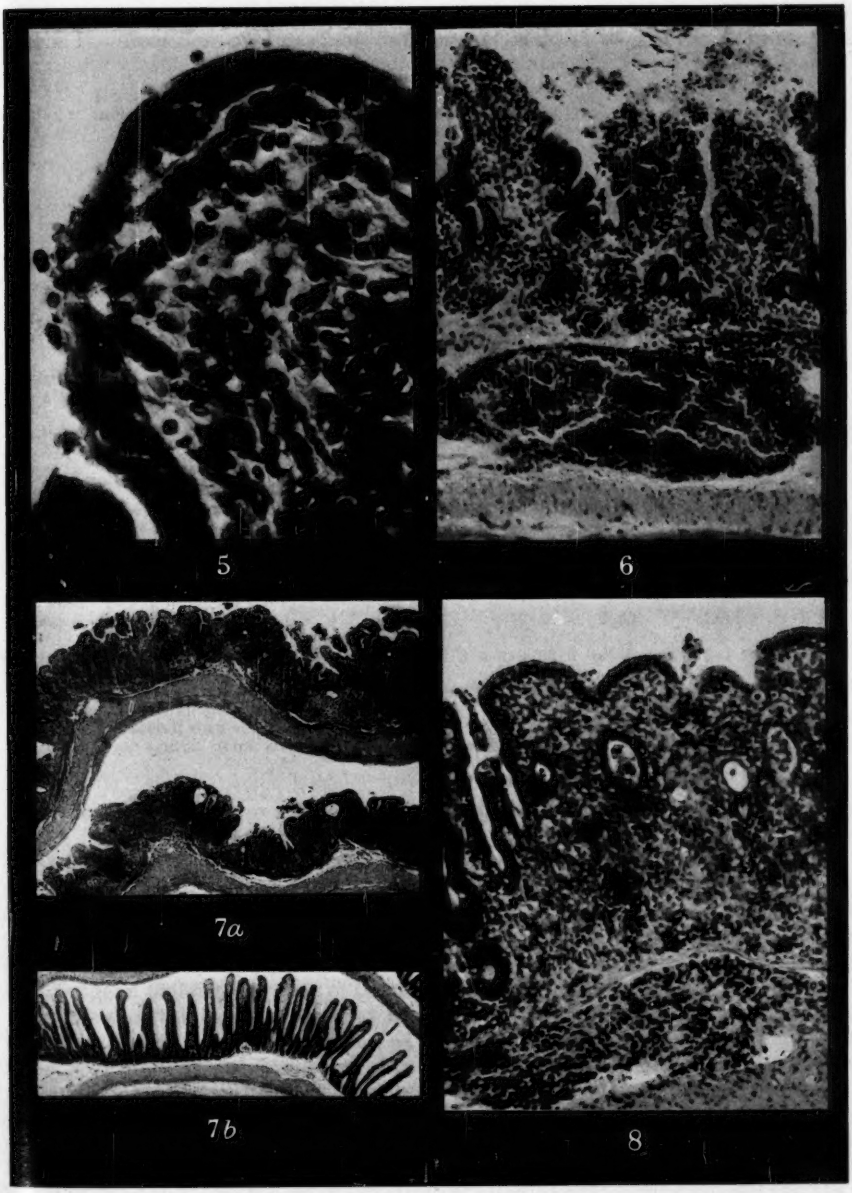
FIG. 7*a*.—Intestinal wall, cholera-infected loop, rabbit ileum, 24 hr. after challenge. Note sprue-like pattern with obliteration of villus architecture. ×22.

FIG. 7*b*.—Non-infected portion of bowel wall adjacent to infected loop from rabbit illustrated in Fig. 7*a*. The general architecture is well preserved. ×22.

FIG. 8.—Higher magnification of intestinal wall illustrated in 7*a*. Note fusion and blunting of villi, severe inflammatory reaction with predominantly mononuclear response in the mucosa and cellular infiltrate of the submucosa with numerous segmented leucocytes.

Formal, Kundel, Schneider, Kunev and Sprinz.





Formal, Kundel, Schneider, Kunev and Sprinz.

TABLE I.—*Condition of Intestinal Segments of Rabbits at Various Times After Infection with* V. cholerae

Hr. after infection	Condition of segment*				Total
	I	I + MN	I + SN	MA	
4–8	57	4	0	2	63
9–12	7	5	7	5	24
13–20	3	0	25	1	29
Total	67	9	32	8	116

* I—Acute inflammation of bowel wall.
I + MN—Acute inflammation with focal necrosis of bowel wall.
I + SN—Severe necrosis of bowel wall.
MA—Sprue-like pattern of bowel mucosa.

TABLE II.—*Lactic Acid in Intestinal Segments of Rabbits at Various Stages of Infection with* V. cholerae

	Acute inflammation		Acute inflammation with focal necrosis		Severe necrosis		Uninfected ligated*	Normal†
	C‡	N§	C‡	N§	C‡	N§		
No. of animals	15	15	7	7	14	14	2	5
Avg. total lactic acid (mg.)	0·4	0·8	4·7	0·8	17·2	2·63	1·7	2·9
Range (mg.)	0·1–1·1	0·3–1·8	0·7–8·1	0·5–2·3	2·0–33·0	0·4–6·4	1·5–1·9	0·4–5·6

* Fluid from above ligated segment in non-infected rabbits.
† Fluid from the small intestine of normal rabbits.
‡ Cholera infected segment.
§ Total lactic acid in 10 ml. sample from normal intestine above the ligated segment.

In segments with no evidence of necrosis the lactic acid content was low. As the infection progressed to bring about mild and later severe necrosis of the intestinal wall the lactic acid content of the segments increased and in loops with severe necrosis reached values comparable to those reported by Jenkin and Rowley (1959). The total protein content of the fluid also increased as the infection progressed. The fluid from most segments with no necrosis contained in the average 49 mg. per cent of total protein while those whose walls were severely necrotic had an average of 620 mg. per cent of total protein.

In view of the observation that in the first stages of the infection fluid from the rabbit intestinal segment appeared in the lumen with little if any increase in lactic acid content, other species were experimentally infected with *V. cholerae per os*. Infant rabbits (10 days old) were infected following neutralization of stomach acid with calcium carbonate ; guinea pigs were infected using a modification of the procedure reported by Freter (1955). The results of these experiments (Table III) indicate that lactate concentration of fluid taken from the caecum of infected infant rabbits and from the small intestines of infected guinea-pigs was not elevated.

Jenkin and Rowley (1959) found that injection of 20 mg. or more of sodium lactate into isolated segments of rabbit ileum produced distention of loops with

TABLE III.—*Lactic Acid Content of Intestinal Fluid From Guinea-Pigs and Infant Rabbits Experimentally Infected with* V. cholerae

	Guinea pig		Rabbit	
	Infected	Normal	Infected	Normal
No. animals tested	10	4	11†	0
Average total lactic acid* (mg.)	0·65	0·8	0·8	Not done
Range (mg.)	<0·02–4·3	0·1–1·6	<0·01–4·7	Not done

* Total lactic acid in the small intestine of the guinea pig and in the caecum of the infant rabbit.
† In 5 of the 11 samples lactic acid could not be detected with the procedure employed.

fluid and haemorrhagic necrosis of the intestinal epithelium with sloughing of the epithelial lining. In their experience, lower doses of lactate could evoke a response if the animal had been previously injected intravenously with endotoxin. Using two different products our results in a larger number of animals than they employed were not consistent. In only a portion of the segments receiving over 50 mg. sodium lactate was there evidence of fluid accumulation and in a given animal a positive loop might be observed with a lower dose of lactate while segments with higher doses were negative.

We were also unable to detect a significant increase in the number of positive loops observed in rabbits injected intravenously with 40 μg. of a *Shigella flexneri* 2a endotoxin 3 hr. prior to the preparation of intestinal segments with sodium lactate. The endotoxin employed was one prepared by the method of Westphal, Luderitz and Bister (1952) and was pharmacologically active in the dose employed ; it induced diarrhoea in approximately 20 per cent of the animals and was lethal for about the same proportion. Most animals were killed 6 hr. after the administration of the lactate. A few animals surviving treatment with both endotoxin and lactate were examined after 20 hr. The results of the administration of sodium lactate alone and with endotoxin are summarized in Table IV. Jenkin and Rowley

TABLE IV.—*Reaction of Rabbit Intestinal Segments Following the Injection of Sodium Lactate*

		Amount sodium lactate injected (mg.)					
		Baxter			Fisher		
Pretreatment	* Time of inspection	19	38	57	16·5	33	66
None	+20 hr.	6/18†	8/31	8/31	1/2	1/4	0/3
Endotoxin‡	+6 hr.	6/12	0/12	—	—	—	—
	+20 hr.	0/5	4/5	0/3	—	—	—

* Number of hours after the injection of sodium lactate.
† Number positive/total injected ; all intensities considered positive.
‡ 40 μg. *Sh. flexneri* 2a endotoxin injected intravenously 3 hr. prior to the preparation of the sodium lactate segments.

(1959) used an endotoxin prepared from *V. cholerae*. While the difference in source of the endotoxins may be the cause of the discrepancy, this is not thought to be the case because the dysentery culture from which our endotoxin came also produced positive loops which contained elevated levels of lactic acid in the late stages of infection.

DISCUSSION

Acute inflammation with inflammatory cellular exudation has been considered not to be a factor in either human cholera or in the experimentally produced disease in animals. The loss of large amounts of fluid in the rice water stool, pathognomonic for cholera, has been attributed to epithelial denudation (De and Chatterjee, 1953 ; Pollitzer, 1959). This conclusion was based on evidence accumulated from human autopsy cases or from animals dying from the disease process. However, Gangarosa, Beisel, Benyajati, Sprinz and Piyaratn (1960) recently have demonstrated in humans that at the height of the clinical disease an active inflammatory reaction and an intact epithelial lining were present. We have shown that *V. cholerae* in the ligated loop of rabbit intestine elicits an acute inflammatory reaction with both vascular and cellular components, but in the late stages karyorrhexis and cytolysis occur and evidence of cellular infiltration may no longer be evident. A similar acute inflammatory reaction also occurs in the small intestine of guinea pigs experimentally infected with *V. cholerae*. In this species many segmented leucocytes are also present in the early stages of the infection but tend to disappear in the late stages of the disease and were not seen in the intestine of animals which died from the infection (Sprinz, Kundel, Schneider and Formal, unpublished).

The experimental infection shares with the human disease its basic pathogenesis : an acute non-specific inflammation with a prominent vascular component. In the special condition of the ligated rabbit loop the process advances to focal necrosis which increases with time. This necrosis follows the acute inflammatory reaction and is in our opinion primarily due to a severe disturbance of the circulation of the intestinal villi leading to vascular stasis and tissue ischemia. The occurrence of necrosis is considered to be a secondary phenomenon which does not invalidate the intrinsic unity of tissue response to *V. cholerae* in both man and the experimental animals.

In the *V. cholerae* infected loop outpouring of fluid into the lumen is associated with an acute inflammation and occurs at a time when neither the lactic acid nor the protein levels are elevated and prior to necrosis. Therefore, it seems unlikely to us that lactic acid plays a role in the production of the cholera inflammation or the cholera diarrhoea as suggested by Jenkin and Rowley (1959). The level of lactic acid does correlate with the severity of necrosis of the bowel wall ; whether elevated lactic acid levels are the cause or result of necrosis of the intestinal wall is not clear but is irrelevant to the discussion of the pathogenesis of the cholera stool, since both occur after the onset of acute inflammation and after the accumulation of fluid in the intestinal loop.

De and Chatterjee after noting a protein-rich fluid in their cholera infected rabbit loop postulated that a protein rich fluid was also formed in the human disease but was later reabsorbed. We would explain the elevated protein content in the fluid of isolated loops of moribund animals by the fact of necrosis. Our work indicates that inflammation is the basic pathogenic process and that although protein rich fluid is seen in the interstices and lymphatics of the lamina propria the intact epithelium serves as a barrier to substances of large molecular weight but permits transit of water and electrolytes. Our morphological studies agree in this regard with physiological investigations of Gordon (1960).

A number of animals developed intestinal lesions exhibiting some of the features of severe human sprue. This is one of the basic histological patterns which result

from the reaction of the intestine to injury. We have observed a similar pattern in man in a case of acute malabsorption similar to that described by King and Joske (1960), and in germ-free and conventional guinea-pigs experimentally infected with *Sh. flexneri*. In addition, Cameron and Khanna (1959) produced a similar lesion by a transient ligation of the superior mesenteric artery, and Fischer (1929) has illustrated this in severe cases of human dysentery.

SUMMARY

The isolated intestinal segment of rabbit ileum was employed as the experimental model for the study of cholera. Infection with *V. cholerae* produced an outpouring of fluid into the gut lumen which was associated with an acute inflammatory reaction and a markedly increased permeability of the capillary bed. Fluid was lost through an anatomically intact epithelial lining. This lining appeared to act as a barrier to the passage of protein. Lactic acid did not appear to be associated with the accumulation of fluid within the segment.

The findings in the early stages of the infectious process in this experimental model resemble those recently obtained in Asiatic cholera of man.

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NUTRITIVE REQUIREMENTS OF A SMALL PLAQUE MUTANT OF WESTERN EQUINE ENCEPHALITIS VIRUS*

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A MUTANT of Western Equine Encephalitis (WEE) virus has been isolated, which forms very clearcut small plaques, allowing titrations as accurate as with the wild type. Its sensitivity to variations in conditions of the nucleic acid metabolism of the cell may make it a useful tool to study some characteristics of this metabolism.

MATERIALS AND METHODS

Virus strains.—WEE virus was obtained from Dr. A. Sabin, in 1956; Newcastle Disease Virus (NDV) was isolated in Belgium by Dr. Florent, and the MEF1 virus was the classical poliomyelitis type II virus.

Culture Media.—Eagle's medium contained double concentration of amino acids and vitamins. Lac-yeast medium consisted of Hanks' solution 99·45 per cent, lactalbumin hydrolysate 0·5 per cent and yeast extract 0·05 per cent; cells were grown in this medium plus 10 per cent calf serum until they were confluent monolayers.

Dialyzed serum was obtained by dialysing 250 ml. amounts of calf serum against 2 changes of 5 l. of phosphate buffered saline, for 48 hr. at 4°.

Structural analogues of nucleic acid precursors were commercially available, except for 5-fluorouracil, which was kindly provided by Hoffman-La Roche Laboratories.

Plaque assay.—Monolayers of chick embryo primary cultures (for the assay of NDV and WEE viruses) or monkey kidney primary cultures (for the assay of poliovirus) were grown in petri dishes 9 cm. in diameter. At infection 1 ml. of the appropriate virus dilution was placed on the plate and after an adsorption period of 1 hr. at 37°, an agar overlay was added. The optimal composition of this overlay was found to vary with the various viruses: 5·4 ml. of agar solution (1·3 per cent) were supplemented with (*a*) in the case of NDV and MEF1 virus, 1·8 ml. 4-fold concentrated Eagle medium (minus bicarbonate) + 1·2 ml. dialyzed calf serum + 0·05 ml. of a 7 per cent bicarbonate solution; (*b*) in the case of the mutant WEE/S, 1·8 ml. 4-fold concentrated balanced salt solution (BSS) + 1·2 ml. non-dialyzed calf serum + 0·05 ml. of the same bicarbonate solution; (*c*) in the case of the wild type WEE/L virus, the same as in (*b*), except that bicarbonate was omitted.

WEE, MEF1 and NDV plaques were read, respectively, after 40 hr., 3 and 4 days; 1 ml. of neutral red 0·075 per cent, adjusted to isotonicity with a concentrated balanced salt solution, was poured on the agar, and the plaques read after 3 hr.

Anti WEE virus serum.—To 2 ml. of allantoic fluid infected with the wild type WEE virus, were added 2 ml. Arlacel A + Bayol F, and the mixture was injected subcutaneously into a rabbit; 7 injections were performed at 3–4 days intervals, and the rabbit was bled a week after the last injection.

RESULTS

Isolation of a small plaque mutant (WEE/S).

When assayed for plaques on chicken fibroblasts, appropriate dilutions of allantoic fluids from chick embryos infected with WEE virus gave rise to large

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plaques (6 mm. diameter), quite homogeneous in size, appearing as soon as 36 hr. after inoculation of the monolayer. Similar plaques were obtained when the virus stock was prepared *in vitro*, by growth on chicken fibroblasts supplemented with Eagle's nutrient medium (with or without calf serum added), or with lac-yeast medium plus 10 per cent calf serum. It was however noticed that when no serum was added to the lac-yeast medium, the virus stocks thus grown gave rise to about 10 per cent small plaques (1 mm. diameter). These plaques were very clear-cut and not of the delayed type, the highest number being found at 36 hr. There were no plaques of intermediary size and for both small and large plaques, plaque morphology was found to be a hereditary character; several plaques of each type were picked and were found to reproduce the same type of plaque on re-plating.

Purification was performed by 3 successive isolations and virus stocks were then prepared by growing the population of one plaque in chicken fibroblasts with one of four media (Eagle's or lac-yeast medium, with or without 10 per cent calf serum); these stocks were then assayed for plaques. Conditions of titration were such that 1 large plaque could be detected in a plate where 800 small plaques were present, while the existence of 1 small plaque was apparent in a population of 100 large plaques. Results obtained from virus stocks derived from large plaques confirmed those previously noted with the wild type population: a small percentage of small plaques were observed when growth had taken place in lac-yeast medium without serum, and none were detected (*i.e.* the proportion of small plaques was less than 0·01) in the case of growth in the 3 other media. On the other hand, virus stocks derived from small plaques and grown in lac-yeast or Eagle's medium contained a few large plaque-formers per 800 small plaque-formers; this proportion increased by a factor of 3–5, when growth had taken place in media supplemented with 10 per cent serum.

As these facts indicated that serum was favourable to the selection of a few large plaque revertants, and that lac-yeast medium was more favourable than Eagle's medium, for the selection of a few small plaque mutants, it was decided to study the nutritive requirements of these two mutants.

The optimum conditions for plaque assay of the mutants were first investigated. It was found that plaque morphology and number of each type of plaques did not vary when the agar overlay (containing 15 per cent calf serum) was adjusted to isotonicity with any one of the three following media: Eagles, BSS or BSS + lactalbumin hydrolysate and yeast extract. In contrast, the optimal concentration of bicarbonate was not the same for both mutants.

Influence of bicarbonate concentration on plaque assay and growth of WEE/S and the wild type WEE/L viruses.

Appropriate dilutions of WEE/L and WEE/S viruses were plated on monolayers of chicken fibroblasts and the agar overlay was supplemented with BSS + 15 per cent calf serum; to this were added various amounts of a 7 per cent bicarbonate solution, to reach final concentrations from 0 to 1·6 per cent. Results in Fig. 1 show that the number of large plaques of WEE/L virus progressively decreases with increasing concentrations of bicarbonate; the higher figure was obtained when no bicarbonate was added (some CO_2 however was supplied under these conditions, as the atmosphere in the incubator is enriched with 5 per cent CO_2). With WEE/S virus, high concentrations of bicarbonate were much less

inhibitory; there was an optimum concentration of 0·4 per cent and this concentration was chosen for routine assay of WEE/S virus; from this experiment on, WEE/L virus was assayed without any bicarbonate added to the agar overlay.

The fact that concentrations of bicarbonate which are optimal for WEE/S are in contrast inhibitory to WEE/L virus, was confirmed in experiments where both viruses were grown separately in liquid medium (Eagle's minus bicarbonate), supplemented with various concentrations of bicarbonate. In this instance, in contrast to the preceding experiments, there was no external supply of CO_2, as the culture tubes were stoppered with rubber. About 1 infective virus particle per 10 cells was introduced into the tubes, and several cycles

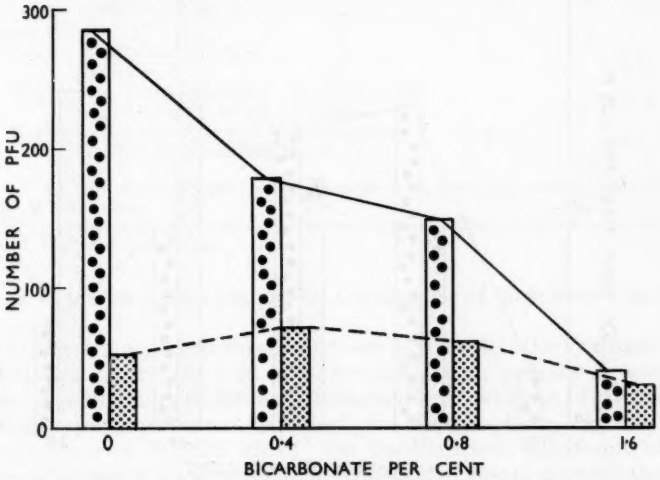


FIG. 1.—The influence of bicarbonate concentration in the agar overlay, on plaque assay of WEE/L (large dots) and of WEE/S (small dots).

of infection were allowed to proceed for 48 hr.; cells and fluid were then submitted to 6 cycles of freezing and thawing, and the virus titres determined. As shown in Fig. 2 optimal bicarbonate concentrations were 0·4 per cent for growth of WEE/S, and 0·1 per cent for growth of WEE/L virus (concentration in normal Eagle medium is 0·275 per cent); when WEE/S mutant was grown in 0·1 per cent bicarbonate, large plaques appeared in the virus stock, in the proportion of 20 per cent. Bicarbonate concentrations as low as 0·05 per cent were insufficient, even for growth of WEE/L virus, either because the pH was unfavourable, or because synthesis of WEE/L, although inhibited by large doses of bicarbonate, was dependent on some bicarbonate supply.

Some other nutritive requirements of WEE/S and WEE/L viruses, when grown in the presence of optimal bicarbonate concentration.

Both viruses were grown in liquid media, under the same experimental conditions as described in the preceding paragraph; bicarbonate concentration was

0·1 per cent for growth of WEE/L virus, and 0·4 per cent for WEE/S. Calf serum, when added, had been dialyzed.

Experiment A in Table I shows that addition of 10 per cent serum to the Eagle's medium increased the growth of WEE/L virus by a factor of 200 while growth of WEE/S was only stimulated by a factor of 2. On the other hand (expt B), lac-yeast medium was twice as favourable to the growth of WEE/S as to growth of WEE/L virus: compared with Eagle's medium, lac-yeast medium was a better nutrient for both viruses, but the increase in titre was 2·5 in the case of WEE/L and 5 in the case of WEE/S.

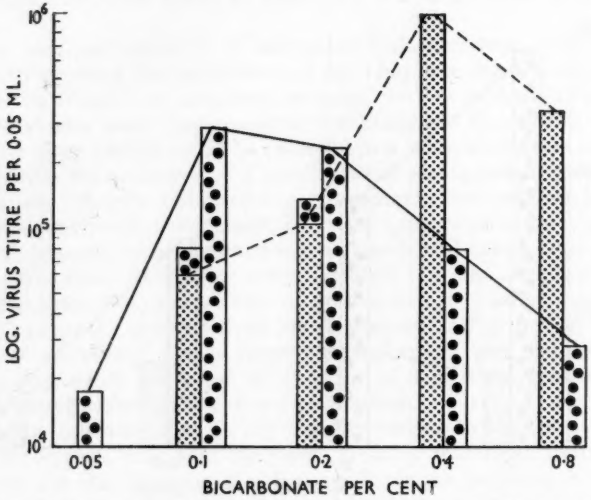


FIG. 2.—Growth of WEE/L (large dots) and of WEE/S (small dots) in liquid Eagle's medium added with various concentrations of bicarbonate. Virus titres in cells + fluid, after 48 hr. at 37°, as determined under optimal plaque assay conditions, for each type of mutant (no bicarbonate added, in the case of WEE/L; bicarbonate 0·4 per cent in the case of WEE/S).

Eagle's media were then prepared, in which one amino acid or one vitamin was omitted. No obvious influence on the growth of the WEE mutants was observed, except in the case of the omission of folic acid: this omission unexpectedly increased the synthesis of WEE/L virus (factors of increase 2·5 and 1·6 respectively in the absence and presence of dialyzed serum), although it decreased growth of WEE/S by a factor of 3.

When amethopterin (0·001 mg. per ml.), was added to Eagle's medium minus folic acid to antagonize the folic function of the cells, growth of WEE/L was still stimulated (factor of increase 1·5), while growth of WEE/S virus was further depressed by a factor of 2.

When all the conditions favourable for the growth of WEE/L were met (*i.e.* Eagle's medium minus folic acid plus amethopterin plus serum, bicarbonate concentration 0·1 per cent), chicken fibroblasts fed with this medium and inoculated with the population of one small plaque gave rise to a virus stock which formed

TABLE I.—*Growth of WEE/L and WEE/S Viruses in Various Media. Preferential Stimulation of WEE/L Synthesis by Calf Serum, and of WEE/S by Lac-yeast Medium. The Importance of the Folic Acid Function for the Growth of WEE/S.*

	WEE/L	WEE/S
Titre in the control medium (Eagle's*)	$1{\cdot}12 \times 10^6$†	$2{\cdot}24 \times 10^7$
Ratio of titres obtained in various media		
EXPT A:		
Eagle's med. + 10 per cent serum‡ / Eagle's med.	200	2
EXPT B:		
Lac-Yeast med. + 10 per cent serum / Eagle's med. + 10 per cent serum	2·5	5
EXPT C:		
Eagle's med. minus folic acid / Eagle's med.	2·5	0·3
Eagle's med. minus folic acid + 10 per cent serum / Eagle's med. + 10 per cent serum	1·6	0·4
Eagle's med. minus folic acid + amethopterin / Eagle's med. minus folic acid	1·5	0·5

* Added with 0·1–0·4 per cent bicarbonate in the cases, respectively, of WEE/L and WEE/S virus.
† Number of PFU per ml., after several cycles of infection for 48 hr. at 37°.
‡ Dialyzed calf serum.

as much as 45 per cent large plaques in the absence of bicarbonate in the overlay (Fig. 3).

Folic acid plays a part in three functions of the cell: the synthesis of glycine, the methylation of uridylic acid into thymidilic acid, and the *de novo* synthesis of purines. A preliminary experiment indicated that addition of glycine (0·05 mg./ml.) to Eagle's medium minus folic acid plus amethopterin, did not restore the optimal synthesis of WEE/S virus. On the contrary, significant observations were made when attempts were made to remedy the defects of either the thymidine or the purine metabolism. This led to the following investigations.

Influence of normal and abnormal nucleic acid precursors on the growth of WEE/S and WEE/L viruses, and a comparison with the action on two other viruses (NDV and poliovirus)

WEE/S and L viruses were grown as above in Eagle's medium minus folic acid + amethopterin + calf serum, and the effect of adding one of the five purine and pyrimidine bases, or their nucleoside, at a concentration 0·25 mM, was studied; (guanine is poorly soluble and was partly in suspension when used). For comparison, two other viruses were also grown under the same conditions (NDV, on chicken fibroblasts, and poliovirus MEF1, on monkey kidney primary cultures).

Figures in Table II represent the ratio of virus titre in the presence of a nucleic acid precursor to the titre in the absence of this precursor. Most striking was the high increase of WEE/S virus titre in the presence of some of the precursors (factor of increase 53 with guanosine, 46 with cytidine, 41 with adenine, 30 with thymidine and 27 with cytosine). Factors of increase were much lower in the case of the 3 other viruses, and in no instance reached 3-fold. The high dependence of WEE/S virus on nucleic acid precursors for growth suggests its use as a sensitive

tool to detect, for instance, whether the cell utilizes a nucleoside more readily than the free base or vice versa. No generalization can be derived from Table II ; the nucleosides thymidine, cytidine and guanosine were more efficient growth

TABLE II.—*Growth of Four Viruses in Eagle Medium Minus Folic Acid + Calf Serum + Amethopterin, Supplemented with Various Normal or Abnormal Nucleic Acid Precursors, at a Concentration 0·25 mM*

	WEE/L*	WEE/S	NDV	MEF1
Adenine	0·6†	41	0·5	2
Adenosine	0·4	10	1	1
Guanine	1·2	15	0·8	1·3
8-Azaguanine	0·2	2·2	0·8	0·6
Guanosine	2	53	0·9	1·1
Cytosine	1·9	27	—	1·7
Cytidine	2·9	46	0·5	2·1
Uracil	0·5	3	0·5	1·6
Thiouracil	2·9	4	2·1	1·5
5-Fluorouracil	1·6	27	1	0·9
Uridine	0·25	0·7	0·8	1·2
Thymine	1	9	1·1	2
5-Bromouracil	1·7	7·7	—	2·3
Thymidine	1·9	30	0·2	1·7
5-Bromodeoxyuridine	2·2	3	1·5	2·1

Results are the average of 3 expts ; figures lacking in this table are those for which the mean deviation was too great.

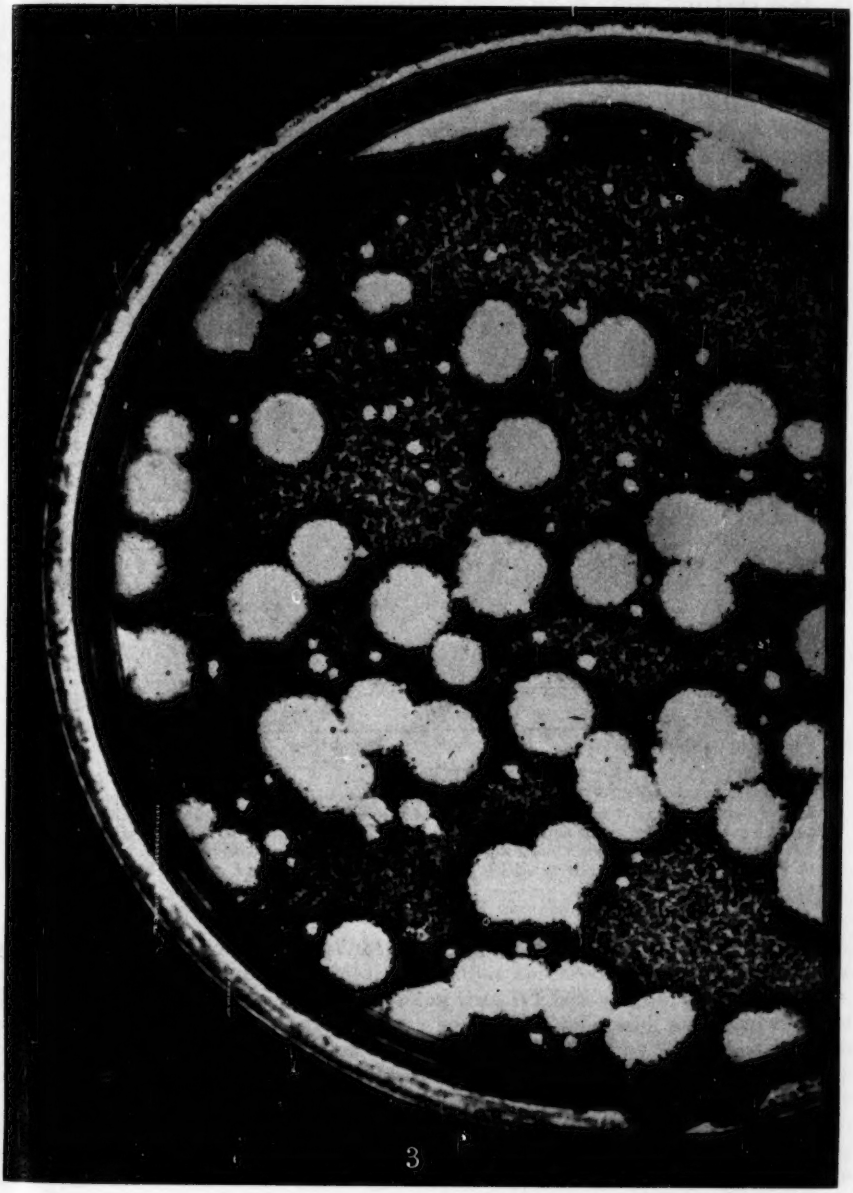
* Ratio of virus titre in the presence of one nucleic acid precursor, to the titre in control medium (without nucleic acid precursor added). Titres in control medium were respectively, per ml., $2{\cdot}48 \times 10^7$ for WEE/L virus ; $6{\cdot}5 \times 10^7$ for WEE/S virus ; $8{\cdot}52 \times 10^6$ for NDV and $2{\cdot}64 \times 10^6$ for the MEF1 strain.

factors for WEE/S virus than the free bases but, in contrast, adenine was more active than the corresponding nucleoside.

When cells were deprived of their capacity to synthesize thymidine, and purine bases, by the presence of amethopterin, structural analogues which have been described to be inhibitory for growth of plant and bacterial viruses (see discussion) were found to increase the synthesis of WEE/S virus : the factor of increase, which was 3 in the presence of uracil, was 4 with thiouracil and as high as 27 with 5-fluorouracil. An analogue of thymine, 5-bromouracil, had nearly the same activity as thymine itself (factors of increase respectively 7·7 and 9). In contrast, 5-bromodeoxyuridine, a deoxyribose product which is known to intervene exclusively in the metabolism of DNA, did not here act as thymidine as a growth factor for WEE/S virus : a slight increase of growth was found with the 4 viruses, in the presence of 5-bromodeoxyuridine (increase of titre from 1·5 to 3), but thymidine was 10 times more active than 5-bromodeoxyuridine towards WEE/S virus, and this was a specific effect, not found with the 3 other viruses. Thus, structural analogues of uracil and thymine, or of their nucleoside, were at least as good growth

EXPLANATION OF PLATE

FIG. 3.—A mixture of small and large plaques, obtained when the mutant WEE/S was grown and assayed under optimal conditions for the selection and expression of large plaque revertants.



Quersin-Thiry.

factors as the normal products themselves, for WEE/S virus, provided that, in the case of the nucleoside, the analogue was of the ribose and not of the deoxyribose type.

Only one structural analogue of purine bases was studied; 8-azaguanine (0·25 mM) was shown (Table II) to be inhibitory for growth of WEE/L virus, and slightly so for NDV and poliovirus, although it increased the formation of WEE/S virus by a factor of 2·2. This rather low increase might be the result of two oppo-

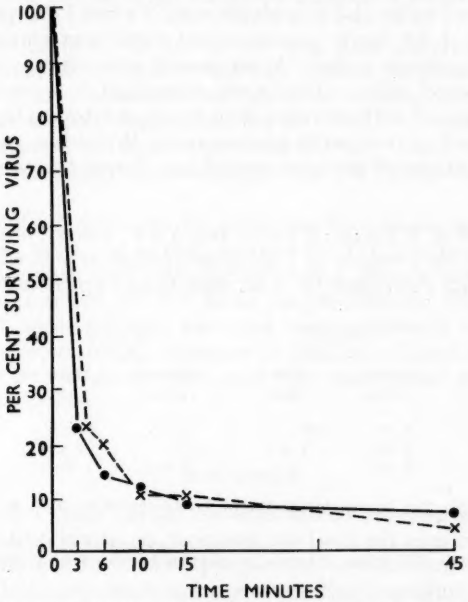


FIG. 4.—Kinetics of the neutralization of WEE/S (———) and of WEE/L virus (– – – –) by anti WEE/L serum. One volume of prewarmed antiserum, diluted 1/30, was added to one volume of prewarmed appropriate dilution of virus. At various times, aliquots were diluted 1/200 in ice cold lac-yeast medium. One ml. of this dilution were plated on each of three monolayers of chicken fibroblasts. The number of PFU, in the controls, was around 70 plaques.

sing effects of 8-azaguanine on WEE/S: a guanine-like growth promoting effect, specific for WEE/S, and an inhibitory action, as observed with the other viruses. In favour of this hypothesis was the finding that WEE/S was not a mutant resistant to 8-azaguanine: when grown in the presence of folic acid, and in absence of amethopterin, WEE/S was inhibited by 8-azaguanine just as WEE/L was (both titres were decreased ten times, as compared with controls, without 8-azaguanine).

When the experiments reported in Table II were repeated, with the only difference that folic acid (0·004 mg./ml.) was present, instead of amethopterin, it was observed that under these conditions, not a single nucleic acid precursor, normal or abnormal, increased the growth of any of the viruses tested; on the other hand, most of them decreased the growth of the viruses, by a factor which ranged from 5–1, an effect which was more accentuated for WEE/S virus.

The synthesis of WEE/L and WEE/S viruses, in cells pretreated with some of the growth factors

Experiments were performed under conditions of a single cycle of viral infection. Culture tubes of chicken fibroblasts were first treated for 6 hr. either with Eagle's medium minus folic acid plus amethopterin (minimal medium), or this medium supplemented with 10 per cent serum, with or without 0·25 mM guanosine or thymidine ; these media were renewed after 3 hr. at 37°. At time 6 hr., the cultures were washed twice and inoculated with 5 virus PFU per cell in 0·3 ml. of minimal medium ; 1 hr. later, non-adsorbed virus was eliminated by washing and 1 ml. minimal medium added. Virus growth was left to proceed for 10 hr. at 37°, after which period cells + fluid were submitted to 6 cycles of freezing and thawing. This series of culture tubes was compared to another series, in which serum, supplemented or not with guanosine or thymidine, was present during adsorption and synthesis of the viruses and not during the pre-treatment period.

TABLE III.—*Growth of WEE/L, WEE/S and NDV, under Conditions of One Cycle of Infection, in the Presence of Calf Serum (alone or with Guanosine or Thymidine), or in Cells Pretreated for 6 hr. with these Ingredients*

Eagle Medium Minus Folic Acid + Amethopterin supplemented with ↓	WEE/L		WEE/S		NDV	
	Before virus	With virus	Before virus	With virus	Before virus	With virus
Serum	1×*	102×	1·5×	10×	0·8×	1·2×
Serum + guanosine	1·5×†	1·8×	11×	3×	0·7×	0·9×
Serum + thymidine	0·25×	1×	2·2×	1·3×	0·9×	0·8×

* Along the first row, the figures relate to the modification of titre, as compared to medium without serum.

† Along the second and the third row, the influence of guanosine or thymidine is emphasized and the figures represent the modifications of titres, as compared with medium plus serum.

As shown in Table III, calf serum increased the growth of WEE/L virus by a factor of 102, when added at the same time as the virus but had no influence when used for pre-treatment of the cells. As already noted the enhancing activity of serum on WEE/S mutant growth was lower than on WEE/L (factor of increase 10) ; with this virus too, the influence of serum depended on its presence during virus synthesis.

A supplement of guanosine, added to the medium plus serum, had small effect on WEE/L growth, but increased the formation of WEE/S virus by a factor of 11, in the case of a pretreatment of the cells, and only by a factor of 3 when added at the same time as the virus. Thus, in contrast to the effect of serum, guanosine is more useful for virus synthesis when it has been stored by the cells first, or has had some effect on the cells. Results with thymidine tend to the same conclusion, although the increase was much lower under the present conditions of one cycle of infection than was found before.

The same experiments were repeated with NDV and MEF1 virus, and disclosed no significant influence of serum, guanosine or thymidine. This comparison indicated that dependence on calf serum for growth was a specific characteristic of both WEE/L and S viruses, the requirement being greater for the wild type

WEE/L ; in addition, the WEE/S mutant was characterized by a supplementary requirement for nucleic acid precursors.

Some other properties of WEE/S mutant

A rabbit antiserum against the wild type WEE virus was assayed against both WEE/L and WEE/S viruses ; the kinetics of the neutralization, a sensitive test (McBride, 1959), did not disclose antigenic difference between the two viruses (Fig. 4).

Thermostability of both viruses at 37° was then assayed. Prewarmed lac-yeast medium was added to each of the two viruses, to make 200 PFU per ml., and left at 37° for 2 hr. ; aliquots were plated at various times. No inactivation of WEE/L virus took place, but infectivity of WEE/S dropped to 66 per cent in 30 min., 50 per cent in 1 hr. and 32 per cent in 2 hr. There were indications too that WEE/S lost infectivity more rapidly than WEE/L, when kept frozen at −70°.

The LD_{50} of WEE/S virus for 10 days old chick embryos, inoculated by the allantoic route, was the same as that found with WEE/L virus ; allantoic fluids recovered from eggs inoculated with WEE/S virus, did not contain more than one or two large plaque-formers, per 800 small plaque-formers. Studies on the pathogenicity of WEE/S virus for mice have not been completed because of an intervening epidemic of ectromelia ; however it has been found that WEE/S virus killed mice by the intracerebral route, and that many small plaque-formers were recovered from the brains.

DISCUSSION

The fact that a few small plaque mutants can be detected in WEE virus stocks grown on chicken fibroblasts, in lac-yeast medium without serum, and the fact that virus stocks derived from small plaques are contaminated with large plaque-formers, if growth has taken place in lac-yeast or Eagle medium supplemented with 10 per cent serum, can best be explained by selective pressures exerted (*a*) by nucleic acid precursors (yeast extract in lac-yeast medium), in favour of a spontaneous small plaque mutant (WEE/S), and (*b*) by calf serum, in favour of large plaque-formers, revertants from WEE/S.

When plaque-purified WEE/S and WEE/L viruses were compared as to their requirements for growth, the addition of 10 per cent calf serum to the medium was found to increase the synthesis of WEE/L virus by a factor of 200, and the synthesis of WEE/S only by a factor of 2. On the other hand, several findings pointed to the importance of optimal conditions in nucleic acid metabolism of the cell, to ensure optimal synthesis of WEE/S mutant. First, the rather high requirement of this virus for bicarbonate—a requirement which cannot be met by controlling the pH with Tris buffer (Fefer, unpublished) ; secondly, a dependence of WEE/S on the folic acid metabolism, as proved by a 3-fold decrease in growth when folic acid was removed from the medium, and a further decrease when this medium was supplemented with amethopterin. The fact that amethopterin, in the absence of an external supply of folic acid, had some effect on the cellular metabolism is of some interest, as it indicates that primary cultures of chicken fibroblasts have at least some folic function of their own, although it has been

reported (Lieberman and Ove, 1960) that this function is poor in primary cultures of rabbit organs, as compared with established mammalian cell lines.

It is to be noted that WEE/L virus is also influenced by the conditions of nucleic acid metabolism : but in the opposite direction from WEE/S virus. Doses of bicarbonate which are optimal for WEE/S growth and plaque formation are inhibitory for WEE/L ; omission of folic acid increases WEE/L synthesis by a factor of 2·5, and a further increase of 1·5 is noted when amethopterin is added.

In the presence of folic acid and of optimal amounts of bicarbonate, the growth of WEE/S virus is not influenced by the addition of purine or pyrimidine bases, or of their nucleosides, to the nutrient medium. A need for these nucleic acid precursors becomes evident when the folic acid function is antagonized by amethopterin : in that case such precursors as guanosine, cytidine or adenine increase the growth of WEE/S by a factor around 50, whereas the factor of increase for WEE/L only ranges from 3–0·6.

The fact that adenine is more effective as a growth factor than adenosine, and cytidine more than cytosine, is consistent with published data (Broda, 1960) which indicate that adenine C14 is incorporated better into ribonucleic acid of rat liver, as the free base, than as the nucleoside,—while the contrary is true for cytosine. However, the same does not hold true for uracil, which was found here to have a low growth promoting action, and uridine none at all, although uridine is incorporated into rat liver ribonucleic acid better than uracil : either the growth promoting effect measured here does not reflect the degree of incorporation of precursors into the cell, or this incorporation is different for chicken fibroblasts cultures from that for rat liver.

The specific increase of growth of WEE/S, in the presence of nucleic acid precursors, may not be an indication that these precursors are directly incorporated into the virus ribonucleic acid : thymine and thymidine also increase the growth of WEE/S, although they are known to be incorporated only in the DNA. Thus, the effect of the precursors may be of a more indirect nature.

Some structural analogues, whose inhibitory actions have been reported in other conditions, can replace the normal nucleic acid precursors, as growth factors for WEE/S virus. (*a*) Thiouracil, an inhibitor of tobacco mosaic virus (Jeener, 1957) and of influenza virus synthesis (Amos and Vollmayer, 1958) was found here, in the presence of amethopterin, to display the same growth promoting activity as uracil. (*b*) 5-fluorouracil was even much more active than uracil. Although fluorouracil inhibits the growth of tobacco mosaic virus, it has been found to be utilized by tobacco leaf to make fully virulent particles, in which as much as one third of the uracil was replaced by fluorouracil (Gordon and Staehelin, 1958). Such an incorporation might take place with WEE/S virus too, but it is hard to explain why fluorouracil should be so much better utilized than uracil. The high growth promoting effect of fluorouracil on WEE/S virus is surprising anyway, in view of the fact that this analogue inhibits the growth of continuous cell lines, due to an interference with DNA metabolism, probably at the site of the thymidine synthetase ; in HEp/1 cells, but not in HeLa cells, there is a second site of inhibition, involving the RNA metabolism (Rich *et al.*, 1958, and 1960). No such data are available concerning primary cultures of chicken fibroblasts. (*c*) 5-bromouracil, an analogue of thymine, provoked the same increase of WEE/S growth as thymine did. Data are lacking too, concerning the way 5-bromouracil is utilized by chicken fibroblasts, but there are indications (Djordjevic and Szybalski, 1960)

that 5-bromouracil as the free base, and in contrast to 5-bromodeoxyuridine, is not incorporated into the DNA of a mammalian cell line, D98. These cells thus seem to behave differently from bacteria, in which 5-bromouracil C14 is freely incorporated into nucleic acids, partly as the abnormal base, partly as thymine, cytosine and uracil C14 (Wacker, Kirschfeld and Weinblum, 1960). As regards the influence of bromouracil on viruses, no effect has been reported on the synthesis of RNAviruses, while as much as 100 per cent of thymine DNA of bacteriophages can be replaced by 5-bromouracil, with a level of 11–13 per cent plaque morphology mutants (Litman and Pardee, 1960). (*d*) 8-azaguanine increased the growth of WEE/S virus by a factor of 2·2, although it was inhibitory for WEE/L, and has been found to inhibit the formation of vaccinia virus (Cogniaux-Le Clerc, 1959). This effect on a DNA virus must be reconciled with all other published data which point to a specific effect of azaguanine on the RNA metabolism, either of *Bacillus cereus* (Chantrenne and Devreux, 1958 ; Mandel and Markham, 1958), or of a mammalian cell line (Hagiwara, 1960). It could be that unnatural RNA is formed in azaguanine treated cells, with a consequent inhibition of the synthesis of viral protein, affecting both DNA and RNA viruses, and that, on the other hand, in cases where there is a marked need for nucleic acid precursors, (*e.g.* for the synthesis of WEE/S mutant) azaguanine could be used by the cell to make viral RNA. Thus, the final titre of WEE/S virus would result from the conflict between inhibition of viral protein formation and promotion of viral RNA synthesis : this would explain why azaguanine is much less active than guanine, as a growth promoting factor for WEE/S, in contrast to analogues of uracil and thymine which are at least as active as the normal base.

Under conditions of a single cycle of virus infection, it was found that enhancement of WEE/S growth by guanosine is not linked to the presence of guanosine in the medium, during virus synthesis, but rather to pretreatment of the cells by this nucleoside ; experiments actually in progress indicate that, to get an optimal virus growth promoting effect, the duration of treatment must be as long as 3–6 hr. Thus, it appears that guanosine needs time to prepare the cell for virus synthesis either through induction of the formation of some adaptive enzyme, or through enlargement of some purine-containing pool. Indications in favour of the existence, in L cells, of adaptive enzymes involved in the conversion of guanine to adenine, have been given by McFall and Magasanik (1960) ; they found on the other hand that incorporation of guanine and adenine C14 into the nucleotide pool is very rapid, a fact not consistent with the long lag observed here between addition of guanosine and the effect on WEE/S virus synthesis : if this effect were to depend on some storage of guanosine, then the incorporation would be related to some remote step of the RNA metabolism, concerning the microsomes, for instance.

SUMMARY

A small plaque mutant of WEE virus has been isolated, with a marked dependence on nucleic acid metabolism for growth. As compared to the wild type, this mutant was found to be more dependent on bicarbonate and folic acid content of the medium ; in the presence of amethopterin, its growth was greatly increased by the presence of some purine and pyrimidine bases, or of their nucleoside ; some structural analogues could replace the normal nucleic acid precursors, as growth factors.

Under conditions of one cycle of infection, the growth promoting effect of guanosine was only found when cells had been pretreated with this nucleoside, before addition of the virus.

In contrast to NDV and poliovirus (MEF1 strain), the wild type WEE/L virus was found to be highly dependent on the presence of calf serum for growth; this property was less accentuated in the mutant WEE/S.

No antigenic differences could be discovered between the 2 WEE variants. WEE/S virus was pathogenic for mice and chick embryos, and bred true *in vivo*. This mutant was more thermolabile than the wild type.

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STUDIES ON THE MECHANISM OF SHOCK

The Activity of the Reticulo-endothelial System after Limb Ischaemia in the Rat

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Interest in the possible role of bacterial products in the response of the body to injury (Fine, 1961) has focused attention on the efficiency of the reticulo-endothelial system (RES) in the injured animal. Because of the importance of this system in the body's defence against both bacteria and their products, schemes in which these products play a significant part in the response to injury include a lowered efficiency of the RES after injury. The latter has been demonstrated after thermal injury in dogs (Warner and Dobson, 1954) and after haemorrhage in rats (Zweifach and Benacerraf, 1958) and dogs (Wiznitzer, Better, Rachlin, Atkins, Frank and Fine, 1960) and has been inferred after these and other injuries in other species. Artificial depression of the efficiency of the RES (" blockade ") increases the sensitivity of animals to such injuries (McKenna and Zweifach, 1956; Zweifach, Benacerraf and Thomas, 1957; Wise, Knecht, Pence and Ondash, 1959; Fine, Rutenburg and Schweinburg, 1959; Fine, Frank, Ravin, Rutenburg and Schweinburg, 1960). Stimulation of the RES, on the other hand, is accompanied by greater resistance to these injuries (Zweifach and Thomas, 1957; Zweifach *et al.*, 1957; Fine *et al.*, 1959; 1960).

The object of the present experiments was to extend this type of investigation to another form of injury (limb ischaemia) in the rat in order to see if the apparent connection between the activity of the RES and the response to injury is a general phenomenon. While maintaining that bacterial products are concerned in the response to haemorrhage in the rat (Friedman, Schweinburg, Yashar and Fine, 1957), Schweinburg and Fine (1960) do not think that they are involved in the rat's response to tourniquet injury which they consider to be a " reversible " form of shock. Their experiments were not a good test since the limb ischaemia used seems to have been a relatively mild injury with the rats surviving more than 18 hr. In the present work two more severe forms of limb ischaemia have been used which led in one case (4 hr. ischaemia) to a " reversible " form of shock and in the other (10 hr. ischaemia) to an " irreversible " form. An irreversible state of tourniquet shock can be defined as one in which death cannot be prevented by giving large volumes of 0·9 per cent NaCl. In both forms of shock the rat's ability to clear its blood of colloidal matter was found to be reduced and the experiments were continued in an attempt to assess the importance of this.

MATERIALS AND METHODS

Albino rats of the Porton strain (body wt. 240 $\pm$ 35 g. S.D.) fed on MRC diet 41b (Bruce and Parkes, 1956) were used. In experiments with a 4 hr. period of limb ischaemia food

was available until the start of the experiment. When a 10 hr. period was used food was removed from the cage on the afternoon before the experiment and coprophagy prevented. Water was freely available. Limb ischaemia was produced as in previous experiments by the method of Rosenthal (1943), a short ether anaesthetic being given for the application of the tourniquets. Control rats were given a similar short period of anaesthesia. Colon temperatures were taken with a mercury thermometer. The environmental temperature was 18–22°.

The following chemical preparations were used. Carbon was given as a colloidal suspension free from shellac (Pelikan Ink C11/1431a; Gunther Wagner, Hanover) as used by Biozzi, Benacerraf and Halpern (1953). For injection this preparation was centrifuged for 20 min. at 1070 *g* to remove any large aggregates and diluted with a 5 per cent (v/v) aqueous solution of polyvinylpyrrolidone (Polyvidone, May and Baker) so that 0·27 ml. of the final suspension contained 8 mg. carbon. Saccharated iron oxide was given as Ferrivenin (Benger Laboratories). This solution contains 20 mg. Fe per ml. and was injected very slowly. Thorium dioxide was given as a stabilized colloidal preparation of 24–26 per cent thorium dioxide in a 25 per cent aqueous dextrin solution (Thorotrast; Testagar, Detroit). Beryllium sulphate was given as an aqueous solution (0·25 mg. Be per ml.) neutralized to about pH 5. Yeast cell membranes were given in the form of zymosan (Zymosan, Type A; Standard Brands, Stamford, Connecticut). Zymosan was prepared for injection by making an 0·5 per cent (w/v) suspension in 0·9 per cent NaCl, heating for 10 min. in a boiling water bath and then exposing the suspension to ultra-sound in a MSE ultrasonic generator at 1·3 amps. The endotoxin preparation used was a lipopolysaccharide prepared from *Shigella dysenteriae* by Dr. D. A. L. Davies of the Microbiological Research Establishment, Porton.

Carbon clearance was determined by a modification of the method of Biozzi *et al.* (1953) on rats lightly anaesthetized with Na pentobarbitone (Veterinary Nembutal; Abbott). Carbon, 8 mg. per 100 g. body wt., was injected into a tail vein and the blood samples were withdrawn from an iliac vein by a needle inserted through the femoral vein. The exponential nature of the fall in the concentration of carbon in the blood was confirmed and routinely 2 samples (0·05 ml.) were taken from each rat. The samples were added to 4·0 ml. 0·1 per cent Na_2CO_3 and compared with suitable carbon standards in an Unicam spectrophotometer S.P. 500 at 675 mμ. Heparin was used as an anticoagulant. The "phagocytic index" (K) was calculated from the formula

$$K = \frac{\log C_{t_1} - \log C_{t_2}}{t_2 - t_1}$$

where C is the concentration of carbon in the blood (mg. per 100 ml.) at time t (min.) The "corrected phagocytic index" (α) was calculated from the formula

$$\alpha = \frac{W}{WLS} \cdot \sqrt[3]{K}$$

where W = body wt. (g.) and WLS = combined weight (g.) of liver and spleen (Biozzi *et al.*, 1953). The theoretical initial concentration of the carbon in the blood (C_{t_0}) was obtained by extrapolation.

Paraffin sections were prepared from tissues fixed in Helly's fluid and stained by routine methods. Blood for smears was collected without anticoagulant in polythene test-tubes after the rat had been guillotined and was transferred to the microscope slide with a polythene tube. The smears were stained with Leishman's stain.

Where possible results are expressed as the mean $\pm$ standard deviation (S.D.) and the means compared statistically by Student's t test as modified by Fisher (1934) for small samples. LD_{50}s were calculated by Weil's method (1952) using groups of 4 animals.

RESULTS

Many of the relevant physiological and metabolic changes following a 4 hr. period of bilateral hind-limb ischaemia in the rat have been described previously (Stoner, 1958*a*, *b*; Stoner and Threlfall, 1960). A 10 hr. period of ischaemia

leads to a more severe state of shock (Haist, 1960), the average survival time being reduced from about 13 hr. to about 4 hr. Whereas the intraperitoneal administration of 12 ml. 0·9 per cent NaCl per 100 g. body weight at the time of removing the tourniquets prevented the death of rats after 4 hr. limb ischaemia it only saved 30 per cent of those given a 10 hr. period.

Clearance of carbon from the blood after limb ischaemia

Shortly after a 4 hr. period of bilateral hind-limb ischaemia the phagocytic index (K) was strikingly reduced. This was accompanied by increases in the "corrected" phagocytic index (α) and in the ratio W/WLS (Table I). The

TABLE I.—*The Effect of Bilateral Hind-limb Ischaemia on the Clearance of Carbon from the Blood of the Rat*

Duration of ischaemia (hr.)	Interval after ischaemia (hr.)	Number of rats	*K*	α Mean ± S.D.	W/WLS
			Fed rats		
0	—	21	0·0421±0·0107	7·12±0·77	20·6±5·7
4	2·25–3·5	10	0·0263±0·0111‡	7·75±0·85*	26·8±2·0‡
4	5–7	5	0·0260±0·0046†	7·48±0·70	25·5±1·2‡
			Fasted rats		
0	—	5	0·0320±0·0079	7·30±0·39	23·2±2·2
10	0·67–1·83	3	0·0143±0·0017†	6·86±0·0017	28·3±1·1*

Dose of carbon = 8 mg. per 100 g. body wt. *K* = phagocytic index, α = "corrected" phagocytic index (see text), W = body wt., WLS = combined wt. of liver and spleen. Environmental temperature 18–22°.

* Significantly different from the control at $P < 0·05$.
† " " " " " " $P < 0·01$.
‡ " " " " " " $P < 0·001$.

change in the latter was due to a very obvious contraction of and expulsion of blood from the spleen, there being no change in the weight of the liver at this stage (Threlfall and Stoner, 1961). These changes were maintained for at least 7 hr. after removal of the tourniquets. Fasting overnight did not affect the phagocytic indices in the controls. A 10 hr. period of ischaemia in these rats led to a greater depression of K without significant change in α (Table I).

Although the intravenous injection of 8 mg. carbon per 100 g. body weight was never fatal in normal rats this dose of carbon sometimes caused death after limb ischaemia. Death was more likely to occur the longer the interval between removal of the tourniquets and the injection of the carbon. In these cases apnoea quickly occurred after the injection and death ensued from respiratory failure.

Distribution of injected carbon after limb ischaemia

In smears of blood taken between 5 and 60 min. after the injection of 8 mg. carbon per 100 g. body weight carbon was found within the platelets and in association with clumps of platelets as described by Stehbens and Florey (1960). In these respects there did not seem to be any significant difference between the injured and normal rats. Most of the carbon, however, was free in the plasma unassociated with cells.

The stability of the carbon suspension in the blood of normal animals has been shown by Stehbens and Florey (1960) and sections of tissues from animals given large doses of carbon (48 mg. per 100 g. body weight) showed that the aggregates formed by fixation were well-dispersed in the blood vessels (Fig. 1). Rats given 8 mg. carbon per 100 g. body weight were usually examined histologically 1 hr. after the injection when there were very few intravascular carbon granules. A different picture was seen after limb ischaemia. While there were very few circulating granules, carbon was found adhering to the endothelial lining of the vessels of the hind-limb (Fig. 2) and also as solid masses plugging some of the smaller ones. Both phenomena were most commonly seen in tissue which had been directly compressed by the tourniquet. Their incidence was reduced, but not eliminated, by giving 200 I.U. heparin per 100 g. body weight intravenously 15 min. before injecting the carbon. Felted masses of carbon were also found obstructing the smaller vessels of the lungs (Fig. 3*a*). In another part of this section carbon was seen incorporated into an antemortem thrombus in a pulmonary artery (Fig. 3*b*). Intravascular agglomerations of carbon were very rarely seen in the lungs of control rats after this dose although they were visible in injured rats killed 5 min. after the injection of carbon.

The histological appearance of the liver in rats killed 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body weight, given 1 hr. after 4 hr. bilateral hind-limb ischaemia, was compared with that of the liver of controls killed at 1 hr. and at 35 min. after this dose of carbon. The average concentration of carbon in the blood of the controls 35 min. after the injection was the same as that at 1 hr. in the injured rats. Although the amount of carbon in the livers of the control and injured rats appeared similar at low magnification (Fig. 4) its distribution was very different (Figs. 5 and 6). In the controls (Fig. 5) the carbon was almost entirely situated within the large Kupffer cells (type G, Lison and Smulders, 1948) both at 35 min. and at 1 hr. after its injection although at the earlier time a few granules appeared to be adherent to the walls of the sinusoids. In the livers from the experimental rats carbon was also found in these large Kupffer cells but they were not as well filled as in the controls. In addition

EXPLANATION OF PLATES

FIG. 1.—Carbon particles in a large hepatic blood vessel of a rat 2 hr. after the intravenous injection of 48 mg. carbon per 100 g. per body wt. H. and E. ×260.

FIG. 2.—Carbon adherent to the endothelium of an artery and vein in the rectus femoris muscle of a rat given 8 mg. carbon per 100 g. body wt. intravenously 1 hr. after 4 hr. bilateral hind-limb ischaemia and killed 1 hr. later. H. and E. ×165.

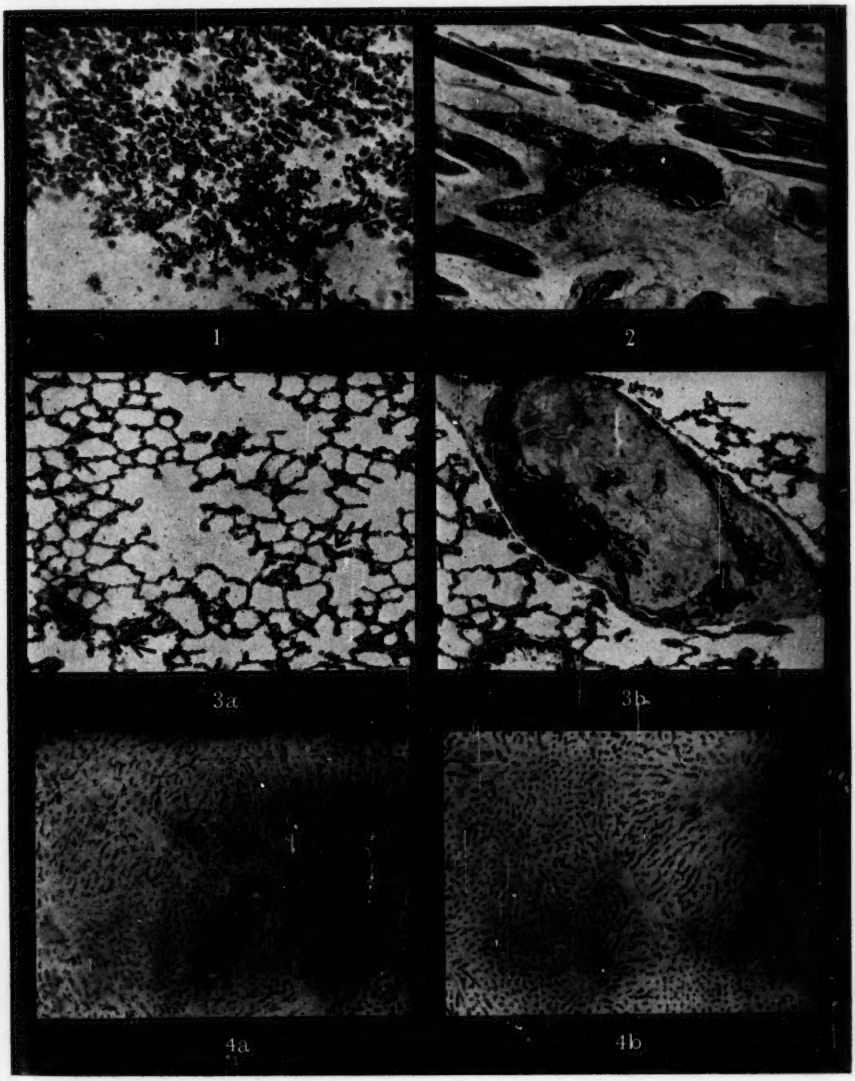
FIG. 3.—(*a*) Carbon masses, shown by arrows, occluding small vessels in the lung. (*b*) Carbon-containing thrombus in a pulmonary artery of the same lung. 8 mg. carbon per 100 g. body wt. injected intravenously 5 hr. after 4 hr. bilateral hind-limb ischaemia and the rat killed 1 hr. later. H. and E. ×165.

FIG. 4.—The distribution of carbon in the liver 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. into (*a*) control rat; (*b*) rat 1 hr. after 4 hr. bilateral hind-limb ischaemia. Unstained. ×66.

FIG. 5.—Carbon in the liver of a control rat (*a*) 35 min. after and (*b*) 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. H. and E. ×530.

FIG. 6.—(*a* and *b*) Carbon in the liver of rats injected intravenously with 8 mg. carbon per 100 g. body wt. 1 hr. after 4 hr. bilateral hind-limb ischaemia and killed 1 hr. later. H. and E. ×530.

FIG. 7.—The distribution of carbon in the spleen 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. into (*a*) control rat; (*b*) rat 1 hr. after 4 hr. bilateral hind-limb ischaemia. Unstained. ×66.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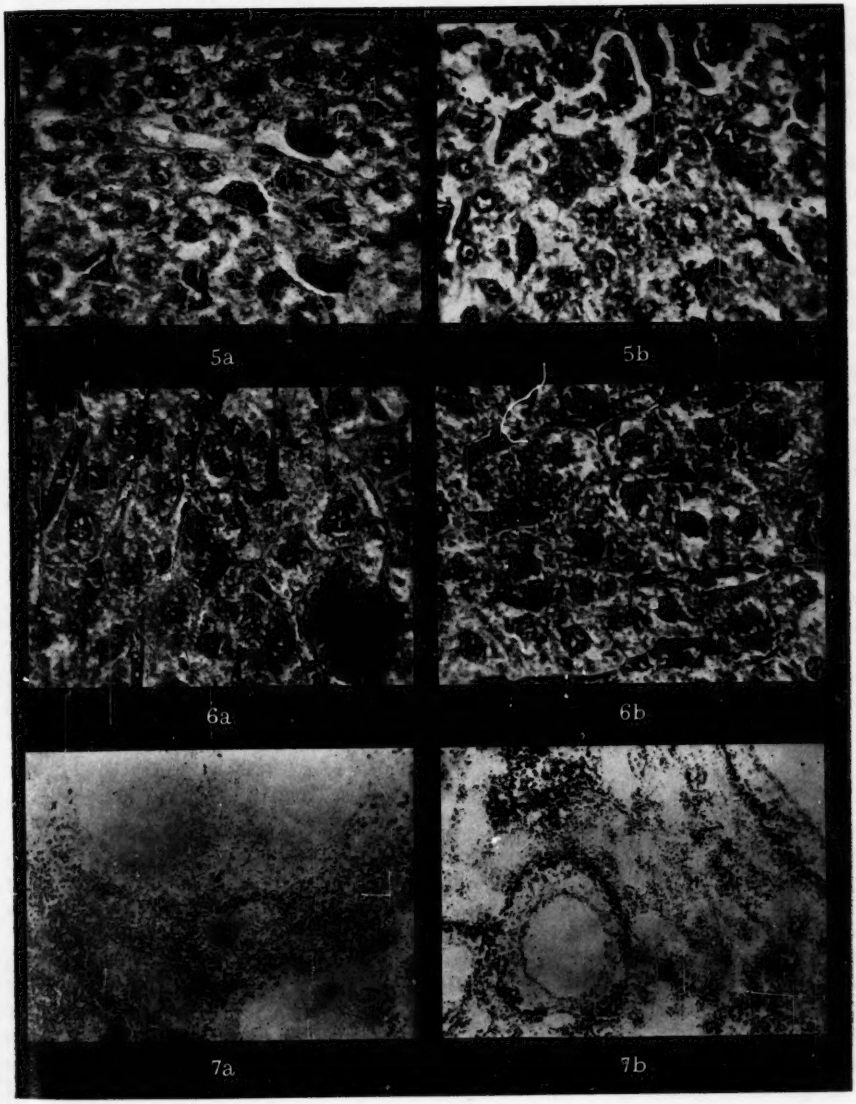
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7a
7b

carbon was also found in the small littoral RE cells (type F, Lison and Smulders, 1948) and considerable amounts of it appeared to be extracellular, adherent to the walls of the sinusoids (Fig. 6). This was reduced but not eliminated by giving heparin as above before injecting the carbon.

Limb ischaemia had a similar effect on the intra-hepatic distribution of saccharated iron oxide (2 mg. Fe per 100 g. body weight) injected intravenously at the same time after removal of the tourniquets.

Despite these changes in the behaviour of the RE cells of the liver towards injected carbon after limb ischaemia it was not possible to detect any certain abnormality in their appearance in haematoxylin and eosin stained sections from injured rats not given carbon and killed at intervals up to the time of death.

The distribution of carbon in the spleens of control rats 1 hr. after the injection of 8 mg. carbon per 100 g. body weight is shown in Fig. 7*a*. The carbon was mainly massed in RE cells in the outer part of the shell of cells surrounding the Malpighian bodies and in cells scattered through the splenic pulp. Under a higher magnification the endothelium of capillaries in the outer part of the Malpighian systems was frequently seen to be studded with carbon granules. After limb ischaemia the spleen contracted and appeared to contain more carbon (Fig. 7*b*). The masses of carbon in the RE cells were larger but the carbon along the endothelial lining of the capillaries was not so obvious as the vessels were contracted.

For comparison the distribution of injected carbon was also observed in rats with the RES damaged by " blockading agents ". Outlining of the liver sinusoids was not a feature after single doses of 48 mg. carbon per 100 g. body weight or when 8 mg. carbon per 100 g. body weight was injected intravenously after " blockade " of the RES with Be. In the spleen after Thorotrast the carbon was displaced from its position around the Malpighian bodies by the " blockading " agent. After beryllium sulphate the appearance of the injected carbon in the spleen was similar to that after limb ischaemia probably because Be causes more damage to the RES of the liver (Cheng, 1956).

Sensitivity to endotoxin after limb ischaemia

The sensitivity of the normal rats to the endotoxin used was rather variable and an exact LD_{50} could not be determined but was about 300 μg. per 100 g. body weight by intravenous injection. An injection of 160 μg. per 100 g. body weight was never fatal and only caused a very slight transient illness.

The administration of Thorotrast (0·6 ml. per 100 g. body weight i.v.) to give RE " blockade " increased the sensitivity so that 2·5 μg. endotoxin per 100 g. body weight intravenously 4 hr. later was a certainly fatal dose. The 1000-fold increase in sensitivity after Thorotrast reported for rabbits by Good and Thomas (1952) was not consistently found in the rat. After carbon (48 mg. per 100 body-weight i.v.) or beryllium (0·025 mg. per 100 g. body weight i.v.) which also " blockade " the RES, the certainly lethal dose was 20 μg. per 100 g. body weight intravenously.

The effects of endotoxin were not altered by large parenteral doses of saline. In 2 groups of 6 rats given 400 μg. endotoxin per 100 g. body weight intravenously one group was also given 12 ml. 0·9 per cent NaCl per 100 g. body weight intraperitoneally 1 hr. later and 4 rats in each group died.

The survival of rats after 4 hr. bilateral hind-limb ischaemia varies from experiment to experiment despite attention to such factors as nutrition, time of day and environmental temperature (Stoner, 1961*b*). Nevertheless, when 2 groups of 8–12 rats are compared at the same time under the same conditions

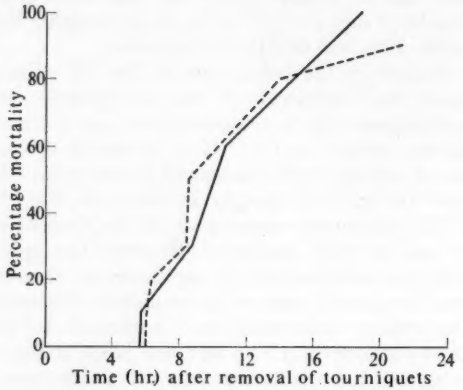

FIG. 8.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of 10 rats. Environmental temperature 18°.

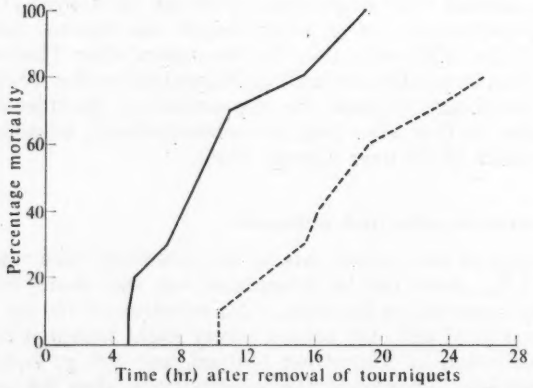

FIG. 9.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of 10 rats, one group (————) being given 100 μg endotoxin per 100 g. body wt. intravenously 1 hr. after removal of the tourniquets. Environmental temperature 20°.

the 2 groups agree in their overall mortality rates, times to 50 per cent mortality and survival times (Fig. 8). Such groups can be used to test the effect of various treatments. Fig. 9 shows the shortening of the survival time after a 4 hr. period of bilateral hind-limb ischaemia by 100 μg. endotoxin per 100 g. body weight given intravenously 1 hr. after removal of the tourniquets. The threshold dose of endotoxin to produce a clinical effect after 4 hr. limb ischaemia was about 20

μg. per 100 g. body weight. Although rats after 4 hr. limb ischaemia were more sensitive to endotoxin, 100 μg. did not shorten the survival time to that commonly seen after 10 hr. bilateral hind-limb ischaemia nor did the rate of the fall in colon temperature during the first 3 hr. after its administration to them differ from that in a group of untreated rats after limb ischaemia.

Effect of " blockade " of the RES on the response to limb ischaemia

The compounds used to " blockade " the RES and their effects on its phagocytic activity are shown in Table II. The doses of Thorotrast, Ferrivenin and

TABLE II.—*The Effect of Compounds Causing " Blockade " of the RE System on the Clearance of Injected Carbon* (8 *mg. per* 100 *g. body weight*) *from the Blood*

Compound	Dose per 100 g. body wt.	Number of rats	K	α Mean ± S.D.	W/WLS
Thorotrast	0·6 ml.	3	0·0216±0·0020†	5·15±0·26‡	18·8±2·7
„ „	0·3 ml.	5	0·0210±0·0088‡	6·06±0·53†	22·8±1·5*
Ferrivenin	0·4 ml.	3	0·0197±0·0135†	5·48±1·20†	21·0±0·3
Carbon	48 mg.	3	0·0286±0·0052*	5·09±0·15‡	18·7±1·3
Beryllium	0·025 mg.	11	0·0199±0·0052‡	5·32±0·57‡	19·7±0·9
Controls	—	21	0·0421±0·0107	7·12±0·77	20·6±5·7

For the significance of K, α and W/WLS see text. The control rats are the same as in Table I.

* Significantly different from controls at $P < 0{\cdot}05$.
† „ „ „ „ „ $P < 0{\cdot}01$.
‡ „ „ „ „ „ $P < 0{\cdot}001$.

carbon used were of the order commonly advised for this purpose. They were given intravenously shortly before the tourniquets were applied so that the phagocytic activity of the RES would be depressed when they were removed after 4 hr. and would remain low for the next 16 hr. *i.e.* during the usual survival period.

After " blockade " of the RES by doses of Thorotrast (0·6 ml.), Ferrivenin and carbon which caused similar depressions of K and α, the mortality rate after 4 hr. bilateral hind-limb ischaemia was increased and the survival time shortened, the 3 compounds producing a similar effect (Fig. 10). This effect of Thorotrast was not reversed completely by giving 12 ml. 0·9 per cent NaCl per 100 g. body weight intraperitoneally when the tourniquets were removed although in the injured controls this therapy was completely effective, reducing the mortality rate from 80 per cent to zero (Fig. 11). A " reversible " form of shock was thus converted to an " irreversible " one by the blocking agent.

A 2 hr. period of bilateral hind-limb ischaemia at an environmental temperature of 18–22° is never fatal in normal rats. When 0·6 ml. Thorotrast per 100 g. body weight was given 4 hr. before the tourniquets were removed after a 2 hr. period of limb ischaemia 3 out of 6 rats died. In a similar experiment with Ferrivenin (0·4 ml. per 100 g. body weight) 7 out of 12 rats died and when the experiment was repeated with 48 mg. carbon per 100 g. body weight 3 out of 9 rats died.

Substances which damage the RES are naturally toxic. The LD_{50}s of Thorotrast and Ferrivenin were approximately 0·8 ml. per 100 g. body weight.

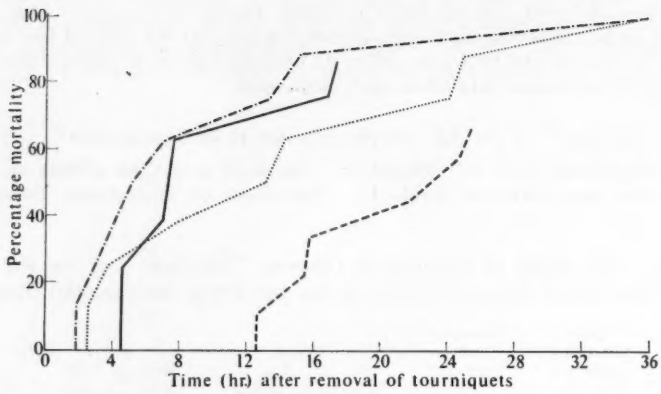

FIG. 10.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 4 groups of rats.

– – – – – – – – Controls (9 rats).
—.—.—. Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. (8 rats).
———— Pretreated with 48 mg. carbon per 100 g. body wt. i.v. (8 rats).
. . . . . Pretreated with 0·4 ml. Ferrivenin per 100 g. body wt. i.v. (8 rats).
Environmental temperature 21°.

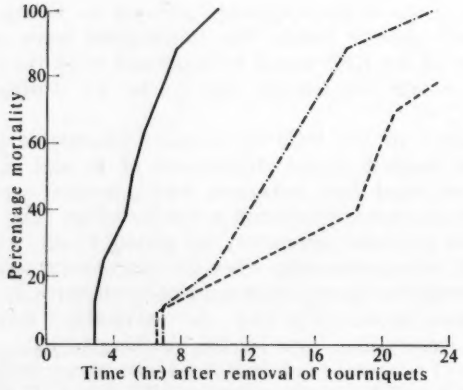

FIG. 11.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 4 groups of rats.

– – – – – – – – Controls (10 rats).
———— Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. (9 rats).
—.—.—. Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. and treated with 12 ml. 0·9 per cent NaCl per 100 g. body wt. i.p. at the time of removing the tourniquets (9 rats).

None of 4th group of 10 rats treated with 12 ml. 0·9 per cent NaCl per 100 g. body wt. i.p. at the time of removing the tourniquets died. Environmental temperature 19°.

The LD_{50} of the carbon suspension was not determined but according to Halpern, Benacerraf and Biozzi (1953) 48 mg. carbon per 100 g. body weight is the maximum single dose which can be tolerated by the rat. It is difficult to damage the Kupffer cells alone for after a time the toxic material they have engulfed diffuses out to the detriment of neighbouring parenchymal cells. In beryllium poisoning in the rat this process has been worked out by Cheng (1956). In this species particulate matter is most actively removed by Kupffer cells associated with the afferent terminal vessels (see Rappaport, Borowy, Lougheed and Lotto (1954) for terminology) and the subsequent changes produce the so-called " midzone " lesion. Although Thorotrast and Ferrivenin in the above doses caused only a transient

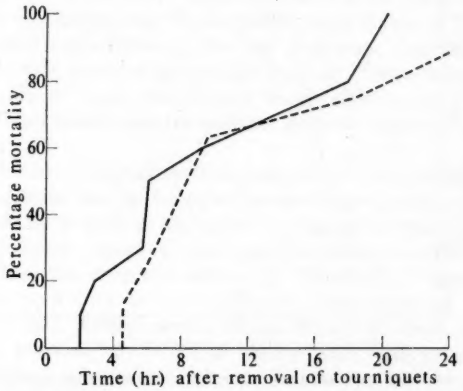

FIG. 12.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of rats.
– – – – – – – – Controls (8 rats).
———— Pretreated with 0·3 ml. Thorotrast per 100 g. body wt. i.v. (10 rats).
Environmental temperature 20°.

outward disturbance in the rat small islets of hepatic necrosis were often found in this area 24–48 hr. later. The appearance and distribution of these lesions were very similar to those produced by endotoxin. Their size was very variable and they appeared reparable. After 48 mg. carbon per 100 g. body weight only occasional necrotic cells were seen near groups of Kupffer cells packed with carbon but carbon granules were also seen within most of the parenchymal cells of the liver.

When the dose of Thorotrast was reduced to 0·3 ml. per 100 g. body weight no parenchymal cell damage was seen during the subsequent 48 hr. This dose reduced K to the same level as 0·6 ml. but the fall in α was less (Table II). In this case the effect on the response to limb ischaemia was relatively slight (Fig. 12). A similar effect was observed in a number of experiments with this dose of Thorotrast.

To obtain a situation where the effect on the parenchymal cells was better defined, RE " blockade " was produced with beryllium sulphate. This has been used for this purpose in mice by Dr. G. E. Paget (personal communication). The dose used (0·025 mg. Be per 100 g. body weight) did not produce any

parenchymal cell damage as judged by the absence of necrotic cells and of regenerative mitoses 48 hr. after the injection. These changes were seen after 0·5 mg. Be per 100 g. body weight. Gross biochemical changes are, of course, required to produce structural damage to the liver cells and even then the histological changes lag behind the chemical ones (Majno, La Gattuta and Thompson, 1960). Nevertheless, experiments in this laboratory with much larger does of Be (Aldridge, Barnes and Denz, 1950; Cheng, 1956; Stoner, 1956) suggest that the parenchymal cells would be little affected by this dose during the 24 hr. following its injection. Although this dose did not visibly disturb the rats its

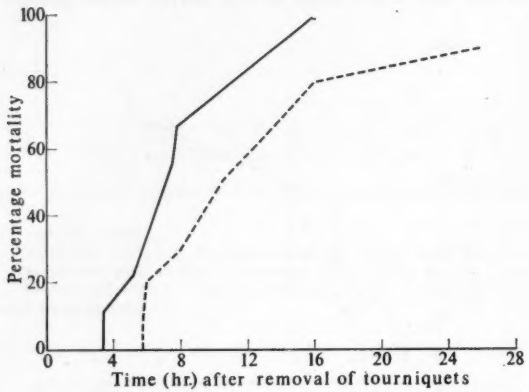

FIG. 13.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of rats.

– – – – – – – Controls (10 rats).
——————— Pretreated with 0·025 mg. Be per 100 g. body wt. i.v. (9 rats).

effect on the RES in respect to carbon clearance is shown in Table II and its effect on the sensitivity to endotoxin has been mentioned above. Blockade of the RES by this dose of Be only moderately increased the effects of a 4 hr. period of bilateral hind-limb ischaemia (Fig. 13).

Effect of stimulation of the RES on the response to limb ischaemia

To increase the activity of the RES with minimal disturbance of other tissues, rats were given 3 daily doses of 1·0 mg. zymosan per 100 g. body weight and used 24 hr. later. Three rats treated in this way had values for K, α and W/WLS of 0·0706 $\pm$ 0·0044, 7·2 $\pm$ 2·5 and 17·5 $\pm$ 0·9 respectively when tested with intravenous doses of 8·0 mg. carbon per 100 g. body weight. The greater activity of the RES after zymosan can lead to anaemia through the increased phagocytosis of erythrocytes (Gorstein and Benacerraf, 1960). Twenty-four hours after 3 daily doses of zymosan the haematocrit was 36 $\pm$ 2 (3 rats) without change in the total blood volume calculated from the C_{t_0} obtained in the carbon clearance experiments. The haematocrit in 13 normal rats was 42 $\pm$ 3 so that there was a significant ($p<0·01$) loss of erythrocytes but it is doubtful if this reduction would seriously impair the oxygen carrying capacity of the blood. Mice repeatedly

injected with zymosan have an increased sensitivity to endotoxin (Benacerraf, Thorbecke and Jacoby, 1959) but this change had not occurred in the rats used for they still tolerated the intravenous injection of 160 μg. endotoxin per 100 g. body weight.

Increasing the activity of the RES in this way had no effect on the response to either 4 or 10 hr. periods of bilateral hind-limb ischaemia.

DISCUSSION

From the results of the carbon clearance experiments it would seem that the phagocytic ability of the RES was depressed after limb ischaemia as after other forms of injury. Comparing these results with those of Zweifach and Benacerraf (1958) in haemorrhagic shock in the rat it is seen that, although the values of K were different, the percentage reductions in " reversible " and " irreversible " haemorrhagic shock were similar to those after 4 and 10 hr. limb ischaemia respectively which could also be said to lead to " reversible " and " irreversible " forms of shock.

However, before a significant depression of the cellular activity of the RES after limb ischaemia can be accepted some other points must be considered.

It is difficult to attach a biological meaning to the expression W/WLS. $\sqrt[3]{K}$ and it will have been observed that the changes in α after limb ischaemia differed from those after the administration of " blocking " agents (compare Tables I and II). This was due to the different behaviour of the spleen in the two conditions. After limb ischaemia it contracted so that, at least after 4 hr. limb ischaemia, the increase in W/WLS more than compensated for the fall in K. This perhaps illustrates some of the artificiality of α.

The phagocytosis of particles by the RES is dependent upon a number of factors a change in which could alter the clearance of carbon without necessarily implying any intrinsic alteration in the RE cells. Among such factors are the blood flow through the RE tissues and the body temperature (Benacerraf, Biozzi, Halpern and Stiffel, 1957 ; Dobson, 1957). The dose of carbon was above the level at which its clearance is determined by the liver blood flow but in the injured rat the failing blood flow to the RE tissues will gradually cease to be compensated by the increased efficiency of extraction from the slower flow and will eventually become a limiting factor. As it has been shown that the liver blood flow after 4 hr. limb ischaemia is maintained within the normal range for some hours (Stoner, 1958*a*) it is doubtful if this would play a significant part at the times studied. It could be more important after the 10 hr. period of ischaemia but data on the blood flow changes under these conditions are not available.

Carbon clearance is affected by changes in body temperature (Halpern, Dick, Biozzi and Mené, 1951) and in experiments on the isolated rat liver with $Cr^{32}PO_4$ Brauer, Holloway and Leong (1957) found that the Q_{10} for the overall reaction of colloid uptake was 1·92. From this it can be calculated that for the results to be explained by the lowered liver temperature after injury it would have to be 30° in the rats studied after 4 hr. hind-limb ischaemia and 21° in those after the 10 hr. period. In the former this temperature would not be reached until 8–10 hr. after removal of the tourniquets (Stoner, 1958*a*) and in the latter the colon temperature at the time of testing had not fallen below 30°.

A further possibility arises from the peculiar kinetics for the clearance of

injected carbon for not only does the carbon concentration in the blood decline exponentially but K × dose = constant, *i.e.* the larger the dose the slower its rate of removal. In the injured rat the effective dose of carbon is increased by the fall in blood volume. The effect of this is shown in Table III and it is seen that this factor alone could not account for the changes observed.

TABLE III.—*The Effect of Alteration in Blood Volume on Carbon Clearance from the Blood*

Controls : Mean initial concentration (C_{t_0} = 114·3 mg. carbon per 100 ml. blood.
Dose (D) = 8 mg. carbon per 100 g. body wt.

Blood volume $\left(\frac{100 \cdot D}{C_{t_0}}\right)$ = 7·0 ml. per 100 g. body wt.

$K \cdot D$ = 0·3368 (fed) ; 0·256 (fasted).

Experimental	Mean C_{t_0} mg. carbon per 100 ml. blood	Equivalent dose (D') of carbon for blood volume of controls	K Calculated $\left(\frac{K \cdot D}{D'}\right)$	K Observed
4 hr. limb ischaemia :				
2·25–3·5 hr. after	124·0	8·68	0·0388	0·0263
5–7 hr. after	126·2	8·83	0·0381	0·0260
10 hr. limb ischaemia :				
0·67–1·83 hr. after	140·3	10·0	0·0256	0·0143

Another point emerged from this exercise. The blood volume of the injured rat can be calculated either from C_{t_0} and corrected for the volume of the injection assuming that it remained in the circulation during the test or from previous data on the haematocrit levels in these rats (Stoner, 1958*b* ; Stoner, Heath and Collins, 1960) assuming that the erythrocyte mass was unchanged. The values obtained by the 2 methods differed, those by the first method being higher (Table IV). This could imply that a fraction of the injected carbon, up to 24 per cent, was rapidly removed from the circulation and did not enter into the determination of carbon clearance. An indication of the fate of this " lost " carbon is given by the histological studies.

TABLE IV.—*Effect of Limb Ischaemia on Blood Volume.*

	Blood volume (ml. per 100 g. body wt.)		
	From C_{t_0} corrected for volume of injection	From haematocrit.	Difference per cent
Control	6·7	—	—
4 hr. limb ischaemia :			
2·25–3·5 hr. after	6·2	5·0	19·4
5–7 hr. after	6·1	4·7	22·9
10 hr. limb ischaemia :			
0·67–1·83 hr. after	5·4	4·3	20

The distribution of the carbon in the tissues of the injured rats differed both from that in the controls and that in rats in haemorrhagic shock (Zweifach and Benacerraf, 1958). Their illustrations show that after severe haemorrhage the uptake of carbon in the liver was restricted to Kupffer cells related to the afferent terminal vessels. Under their conditions the other Kupffer cells seem to have been damaged in the hypotensive episode. After limb ischaemia Kupffer cells throughout the liver contained carbon although in reduced amounts. The distinguishing feature after limb ischaemia was the large amount of carbon associated with the walls of liver sinusoids and of blood vessels in the lungs and elsewhere. This was not observed after haemorrhage by Zweifach and Benacerraf (1958). By light microscopy much of this carbon appeared extracellular but electron microscope studies would be required to decide its precise location. This is thought to be the " lost " carbon mentioned above.

This appearance was not due to precipitation of circulating carbon in fixation since there was very little circulating at the time and it was not seen in controls killed when the concentration of carbon in their blood was the same as in the injured rats (Fig. 5*a*). The effect was not due to the overloading of the rat with carbon. Benacerraf, McCluskey and Patras (1959) have described the attachment of carbon to the vascular endothelium in mice which were either given very large amounts of carbon or injected with carbon after the RES had been extensively damaged with " blocking " agents. Their results suggest that in the present experiments the dose of carbon would be too low and the depression of the RES too slight to give overloading. The effect was not seen in the rats pretreated with Be. Benacerraf *et al.* (1959) have also shown how this appearance can be provoked by giving adrenaline before the carbon. They attributed this to a change in the coagulability of the blood. The appearance of the carbon masses particularly in the lungs of the injured rats certainly suggested a thromboembolic process. Hyperadrenalinaemia was almost certainly present in these rats and it has also been shown that extracts prepared from the ischaemic hindlimb muscles have increased clot-promoting activity (Quastel and Racker, 1941 ; Stoner and Green, 1947, 1951). The deaths which occurred among the injured rats shortly after the injection of carbon are thought to have been due to pulmonary embolism. Their manner of death was the same as that described by Green and Stoner (1947) and rats are known to be very sensitive to the lethal action of pulmonary emboli after limb ischaemia (Green and Stoner, 1950 ; Whiteley, 1954). Limb ischaemia appears to reduce the stability of the colloidal suspension of carbon in the blood as do various clotting factors (Halpern *et al.*, 1953).

From the work of Warner and Dobson (1954) it was reasonable to find apparently more carbon than normal in the spleens of the injured rats. However, this may, in part, be an optical illusion due to the contraction of the spleen. Attempts to determine the percentage of the injected dose in the spleen gravimetrically by the method of Halpern, Biozzi, Mené and Benacerraf (1951) were unsuccessful. The apparent recovery of carbon from the liver, spleen and lungs always exceeded the amount injected and no way of further purifying the extracted carbon was found. This is hardly surprising in view of the adsorptive properties of finely divided carbon. Consequently the carbon in the spleen could not be determined. However, a more important point was that after limb ischaemia the carbon was not displaced from the cells around the Malpighian bodies.

Since carbon attached to vascular endothelium is effectively cleared from the circulation the observed values of K may overestimate the phagocytic ability of the RES after limb ischaemia. The results support the recent criticism of this method by Stehbens and Florey (1960). In a pathological situation of this type it only gives qualitative information and might even be misleading.

A precise explanation of the slower removal of colloid from the blood after limb ischaemia cannot be given. A number of factors have been shown to be incapable of producing the effect single-handed but it is difficult to assess their combined action and so determine the influence of limb ischaemia on the functional ability of the RE cells themselves. Interference with the latter could arise through their metabolic derangement or through their saturation by phagocytosed material and no conclusion can be reached on this.

However, from the practical standpoint the fact remains that after limb ischaemia the rat is not able to remove foreign colloidal material from its blood stream at the normal rate and such material could then be expected to exert an enhanced effect. From the point of view of the endotoxin theory of shock there seems little reason for isolating tourniquet shock in the rat from haemorrhagic shock in that species or tourniquet shock in other species (Schweinburg and Fine, 1960). However, the importance of such material in the response seems small. Further depression of the RE activity increased the response but the effect was relatively slight when parenchymal liver damage was avoided. The hepatotoxic action of the agents used to " blockade " the RES has attracted little attention from their users but doses of Thorotrast which do not cause visible damage produce biochemical changes (Fisher and Fisher, 1961). Even if the effect observed in the experiment of Fig. 13 was not caused by undetected liver damage it need not be due to the action of bacterial products but could represent failure to remove embolic blood clot or fat to which these rats are sensitive. Whatever the nature of the material concerned the normal role of the RES under these conditions would seem relatively unimportant since increasing its phagocytic capacity had no effect. Further experiments are planned to see if the depression of RE function after limb ischaemia assumes greater importance under other experimental conditions as, for instance, when the intestinal flora is altered by dietary changes. While the experiments described in this paper do not investigate the endotoxin theory directly their results do not offer much support for it.

SUMMARY

The effect of hind-limb ischaemia on the functional activity of the reticulo-endothelial system has been studied and an attempt made to assess its importance in the response to this injury in the rat.

As judged by the ability of the rat to clear its blood stream of injected colloidal carbon the phagocytic activity of the reticulo-endothelial system was depressed by hind-limb ischaemia. The degree of depression could not be assessed accurately. In the normal rat the injected colloid remained stable in the blood stream but after limb ischaemia a significant fraction of the dose rapidly became attached to the walls of liver sinusoids and blood vessels in the hind-limbs and obstructed blood vessels in the lungs.

In confirmation of this reduced activity of the reticulo-endothelial system

doses of bacterial endotoxin without effect in normal rats shortened the survival time after limb ischaemia.

" Blockade " of the reticulo-endothelial system by a number of agents also shortened the survival time after limb ischaemia but the effect was not gross if damage to the parenchymal cells of the liver was avoided.

Increasing the phagocytic ability of the reticulo-endothelial system had no effect on the survival time after limb ischaemia.

It is concluded that under the experimental conditions used the reticulo-endothelial system did not play a very important part in the rat's response to limb ischaemia. The value of the carbon clearance method for assessing reticulo-endothelial activity in pathological conditions is also discussed.

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SURVEY OF PAPERS

SCHRIFT, FEIN and SCHAIN transferred various strains of *Corynebacterium diphtheriae* from iron deficient media to media containing known quantities of ferrous iron, sufficient for aerobic growth and pellicle formation. Transfer of the pellicle to medium containing insufficient iron for aerobic growth led to toxin production (p. 399).

EBBA LUND, LYCKE and SOURANDER irradiated cultured cells (HeLa or Detroit-6) and toxoplasms with 600–3000 r X-rays or 30,000–120,000 rads gamma rays. Gamma irradiated parasites penetrated host cells but failed to multiply. Gamma or X-irradiated cells supported parasite multiplication normally. Irradiation of already parasitized cells inhibited parasite multiplication (p. 404).

BRUMFITT and GLYNN, using preparations of rat macrophages and polymorphs obtained from the peritoneal cavity, and human polymorphs separated from blood, studied the fate after phagocytosis of organisms of different variants of *Micrococcus lysodeikticus* Viable counts and counts of visible bacteria after increasing periods of intracellular incubation in either sort of leucocyte showed that lysis and killing were concurrent, and that the lysozyme resistant strain was neither lysed nor killed unless first de-acetylated. Extracts of the two kinds of leucocyte produced lysis curves with sensitive organisms similar in shape to those produced by certain concentrations of egg-white lysozyme, but the lytic property of the cell extracts was not inhibited by anti-egg-white-lysozyme rabbit-serum (p. 408).

OH has made cultures of rabbit corneal endothelial cells by laying Descemet's membrane on plasma-coated cover-slips and incubating them in Leighton tubes. These cultures were infected with egg-grown Newcastle disease virus, and subsequent events studied by staining the cultures with H and E or by the indirect fluorescent antibody technique ; and by the titration of the culture fluids for infectivity by plaque counting on chick-embryo monolayers. He found that the virus caused giant cells and cytoplasmic inclusions to appear in the cultures. Specifically staining antigenic material was also found in the cytoplasm. The virus multiplied convincingly in the cultures (p. 424).

BOULTER, MABER and BOWEN infected rabbits with rabbit-pox (Utrecht strain) and noted pyrexia within 3–6 days and then diminished intake of food and fluid, falling blood-pressure and rising blood urea and plasma potassium. The degree and duration of the hypotension seemed to determine the animals' fate. Only when this was mild and brief did they recover. Pressor amines given before or after the onset of hypotension were without effect on mortality. Corticosteroids were very slightly protective. Attempts have not yet been made to depress the high terminal plasma potassium levels, which were about the same as those causing cardiac arrest in experimental potassium poisoning (p. 433).

KATO, SHIMIZU and HARA showed that a substance in influenza virus (PR8) was active both in a preparatory and provocative role in causing the Shwartzman phenomenon in rabbits. This activity was enhanced by sonic vibration or freezing and thawing of the virus suspension, was separate from infectivity and to some extent from haemagglutinating capacity. The histological appearances of the skin lesions were those usual for the phenomenon (p. 445).

LEIBOWITZ, MORGAN, BERKINSHAW-SMITH and PAYLING WRIGHT produced the Forssman "carotid syndrome" in guinea-pigs by the centripetal injection of the Forssman serum into the ligated right common carotid artery. Three types of antisera, anti-sheep red cells, anti-dog lung, and anti-pneumococcus, were used.

The site of injection in the brain was determined by the obstruction of the arterial blood supply, especially the vertebral artery. The chief injury recognisable histologically falls on the vascular endothelium and this results in local cerebral oedema and haemorrhage.

The degree of damage produced by the heterophile antiserum was also determined with the aid of previously injected ^{131}I-labelled homologous serum. This investigation proved more sensitive than the histological examination ; in some cases there was an increase in the amount of labelled protein in the brain in the absence of any morphological changes and this is probably due to the escape of exudate from the blood vessels in the experimental area of the brain (p. 455).

BRENT, MEDAWAR and RUSZKIEWICZ studied the antigens of the lymphoid cells of strain A mice and were particularly interested in the relation between those which enhance homograft rejection and those which induce recipients of another strain (in this case usually CBA) to form agglutinins and lysins for strain A erythrocytes. They found that these two antigenic characters of their extracted cell free material resembled each other closely in their concentration in various extract fractions and in their resistance to physical and chemical agents, except in two, perhaps unimportant, respects (p. 464).

HOWARD, MICHIE and SIMONSEN studied the graft-versus-host reaction to determine whether the splenic enlargement which occurs is due to an increased number of donor or host cells. (A × C57BL) F1 hybrid mice were injected i.p. with spleen cells from adult A mice. After a week the enlarged spleens of the hybrids were used as a source of cells to inject either isologous hybrids or mice of A × SW parentage sufficiently immunologically mature to eliminate (A × C57BL) F1 cells but vulnerable to A cells. In neither of these procedures did the spleens of the test mice become enlarged, indicating that there were insignificant numbers of A cells in the enlarged spleens of the (A × C57BL) F1 mice affected by the graft-versus-host reaction and that the enlargement was caused by multiplication of their own splenic cells (p. 478).

SCHOENBERG, PRISCILLA GILMAN, MUMAW and MOORE injected rabbits i.v. with polystyrene latex particles (PLP). Clearance of particles from the blood was measured by periodic examination of samples in a phase contrast chamber; and distribution of particles in the tissues was examined by killing animals at successive time intervals and examining sections of Aquon-embedded blocks, both by light microscopy after staining and with the electron microscope. The authors found that neither particle size, dose, nor number of doses influenced the pattern of clearance and distribution, that most of the PLP were in the spleen, and that only a few of the phagocytes, both there and in the liver, contained them (p. 486).

PULVERTAFT, DEBORAH DONIACH and ROITT, using tissue culture techniques, investigated the cytotoxic factor in Hashimoto's disease and its incidence in other thyroid diseases. Trypsinized human thyroid cells in monolayer cultures are killed by Hashimoto sera in the presence of complement. If the complement is inactivated, the cytotoxic effect is abolished. The factor is species and organ specific and is strongest in Hashimoto's disease but is also present in lower concentration in about two-thirds of thyrotoxic sera and in some other thyroid diseases (p. 496).

FORMAL, KUNDEL, SCHNEIDER, KUNEV and SPRINZ used ligated loops of rabbit intestine to study the effects of infection with *Vibrio cholerae*. Histological examination of gut wall at increasing intervals after infection showed that the classical signs of acute inflammation with outpouring of fluid into the gut lumen preceded mucosal necrosis and the accumulation of lactic acid and protein in the lumen. This suggests an explanation for the rice-water stool of the human disease different from that hitherto accepted (p. 504).

LISEQUERSIN-THIRY isolated a mutant from Western Equine Encephalitis (WEE) virus which caused much smaller plaques in chicken-embryo primary monolayer cultures than did the wild type WEE virus. The appearance of the mutant was favoured by the presence of folic acid and high bicarbonate concentration, and reversion to wild type by calf serum and low bicarbonate concentration. In the presence of amethopterin, growth of the small plaque mutant was enhanced by some purine and pyrimidine bases or their nucleosides, and by some structural analogues of such nucleic acid precursors. No antigenic differences were found between this mutant and the large plaque, wild type virus (p. 511).

STONER, continuing his studies on the mechanism of shock, has investigated the activity of the reticulo-endothelial system following hind-limb ischaemia in rats. He found that the phagocytic activity of the RES was depressed in these circumstances. Blockade of the RES shortened the survival time following hind-limb ischaemia but the effect was not marked if damage to the parenchymal liver cells was avoided. He concluded that the RES did not play a major role in the animals' response to limb ischaemia (p. 523).